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Na^+-DEPENDENT, CONCENTRATIVE NUCLEOSIDE TRANSPORT IN RAT MACROPHAGES

SPECIFICITY FOR NATURAL NUCLEOSIDES AND NUCLEOSIDE ANALOGS, INCLUDING DIDEOXYNUCLEOSIDES, AND COMPARISON OF NUCLEOSIDE TRANSPORT IN RAT, MOUSE AND HUMAN MACROPHAGES

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Abstract—The effects of natural nucleosides and various analogs thereof on Na^+-dependent, concentrative transport of formycin B by cultured rat macrophages were investigated. Concentrative transport is the sole nucleoside transport system of these cells. The results indicated that uridine, 5′-fluorouridine, all natural purine nucleosides, 2-chloroadenosine and 5′-deoxyadenosine are efficient substrates for the transporter. None of nine other pyrimidine nucleosides was transported. 3′-Deoxyadenosine, 2′,3′-dideoxyadenosine, 8-azidoadenosine, tubercidin, 5′-methylthioadenosine 6-mercaptopurine riboside and adenosine arabinoside were either poor substrates or not transported significantly. The substrate activity of some of the natural nucleosides and the lack of substrate activity of 3′-deoxyadenosine, 2′,3′-dideoxyadenosine, 8-azidoadenosine and 2′,3′-dideoxycytidine were confirmed by direct uptake measurements. No significant concentrative nucleoside transport was detected in cultured human monocytes/macrophages, whereas mouse macrophages possessed both concentrative and equilibrative nucleoside transporters.

Except for erythrocytes from certain species, all mammalian cells investigated express an equilibrative nucleoside transport system with broad substrate specificity [1, 2]. Na^+-dependent, concentrative nucleoside transport was first detected in epithelial cells of the kidney and intestine and brush border membranes thereof [3–7] but has been found recently to be also expressed, along with equilibrative nucleoside transport, in a variety of other cells [8–12]. In fact, concentrative nucleoside transport has been detected in all types of mouse cells that have been investigated but not in lines of human HeLa and T-cells, of simian embryonic kidney cells, of *Mus dunni* cells and of Chinese hamster ovary cells [10, 11]. In various mouse cell lines concentrative nucleoside transport represents only a minor component of the overall nucleoside transport activity of the cells, whereas in mouse B and T lymphocytes and macrophages concentrative and equilibrative nucleoside transport are expressed at about equivalent levels [8–12].

Rat macrophages are the only cell type identified so far that expresses only Na^+-dependent, concentrative nucleoside transport in the absence of detectable levels of equilibrative nucleoside transport [11]. Thus, concentrative nucleoside transport could be characterized in these cells without interference by equilibrative nucleoside transport. The concentrative system was shown to transport uridine and the natural purine nucleosides guanosine, inosine and 2′-deoxyadenosine but not thymidine or deoxycytidine [11], similar to one of the concentrative nucleoside transporters of mouse intestinal epithelial cells [6]. Concentrative nucleoside transport is highly resistant to various inhibitors of equilibrative nucleoside transport, such as nitrobenzylthioinosine (NBTI) and dipyridamole [3–12]. Exclusive Na^+-dependent, concentrative nucleoside transport, however, is not a general property of rat cells. Normal rat kidney cells express both concentrative and equilibrative nucleoside transport, whereas rat lymphocytes, erythrocytes and Novikoff hepatoma cells express only equilibrative nucleoside transport [11, 12].

Other studies have shown that dipyridamole greatly enhances the salvage of 2′-deoxyadenosine by L1210 mouse leukemia cells which results in increased cytotoxicity of the nucleoside for the cells [13]. This effect seems limited to cells expressing both concentrative and equilibrative nucleoside transport and results from the inhibition by diyridamole of the efflux via equilibrative transport of the deoxyadenosine that is actively transported into the cells [10, 11]. This synergistic effect of a toxic nucleoside and an inhibitor of equilibrative nucleoside transport is of potential interest in chemotherapy directed specifically against cells that express both concentrative and equilibrative nucleoside transport. Obviously it would be limited to nucleosides and their analogs that are substrates for the concentrative nucleoside transporter. Therefore, we have further examined the substrate specificity of the concentrative nucleoside transporter of rat macrophages in relation to various cytotoxic nucleoside analogs. We have also compared nucleoside transport in rat, mouse and human

macrophages in attempts to elucidate the mechanism by which dipyridamole potentiates the inhibition of 2',3'-dideoxycytidine on the replication of human immunodeficiency virus (HIV) in human monocyte/macrophage cultures [14].

MATERIALS AND METHODS

Macrophage culture. Adult female rats (retired breeders; Sprague–Dawley) and adult Swiss mice were obtained from Biolabs, Inc. (St. Paul, MN) and were injected intraperitoneally with 10–20 mL or 2–3 mL of thioglycollate medium, respectively. Two to four days later peritoneal macrophages were harvested from these animals and cultured in 24-well tissue culture plates (2 cm^2/well) with RPMI plus 10% (v/v) fetal bovine serum and 10% (v/v) L-cell conditioned medium (as source of colony stimulating factor 1; CSF-1) as described elsewhere [11, 15]. The cultures were used for transport assays after 1–2 days of incubation with one medium change. Human monocytes/macrophages were isolated from 40 mL of peripheral blood by differential centrifugation in Hypaque/Ficol [16, 17] according to the procedure described by the supplier of the Mono-Poly Resolving Medium (Flow Laboratories, Inc., McLean, VA). The monocyte/macrophage containing gradient fraction was diluted 5- to 10-fold with RPMI and the cells were collected by centrifugation. The cells were washed once in RPMI and then suspended to 1×10^6 cells/mL in RPMI supplemented with 15% (v/v) fetal bovine serum, 10% (v/v) human serum and 10% (v/v) L-cell conditioned medium. Cells were propagated in 24-well plates with repeated changes of the complete growth medium.

L1210 cells. L1210 cells were propagated in suspension culture as described previously [13]. For transport experiments, the cells were collected by centrifugation and suspended in RPMI.

Nucleoside uptake measurements. The medium was removed from cultures in 24-well plates, and the cell layers were rinsed with RPMI and then overlayed with 0.5 mL of RPMI at room temperature (about 25°). Nucleoside uptake was then measured by adding, at timed intervals, a ^{3}H-labeled nucleoside to the desired concentration to 6 wells in a plate. At the completion of the incubation, the medium was dumped out and the wells were rinsed rapidly three times (within 15 sec) with ice-cold balanced salt solution (BSS) as described previously [9–11]. In competition experiments, unlabeled nucleosides were added simultaneously with [^{3}H]formycin B used as a substrate. Cell densities were estimated by counting the cells in several fields of an inverted microscope and converting this number to cells/well on the basis of the areas of the microscopic field and the well [18]. Suspensions of L1210 cells were supplemented with [^{3}H]formycin B and, at appropriate times of incubation, the cells from 0.5 mL of suspension were collected by centrifugation through an oil layer and analyzed for radioactivity as described previously [10].

Materials. ^{3}H-Labeled nucleosides were purchased from Moravek Biochemicals (Brea, CA) and diluted to the desired specific radioactivity with unlabeled substrate. Unlabeled nucleosides were obtained from the Sigma Chemical Co. (St. Louis, MO). Dipyridamole (Persantin) was a gift from Geigy Pharmaceuticals (Yonkers, NY).

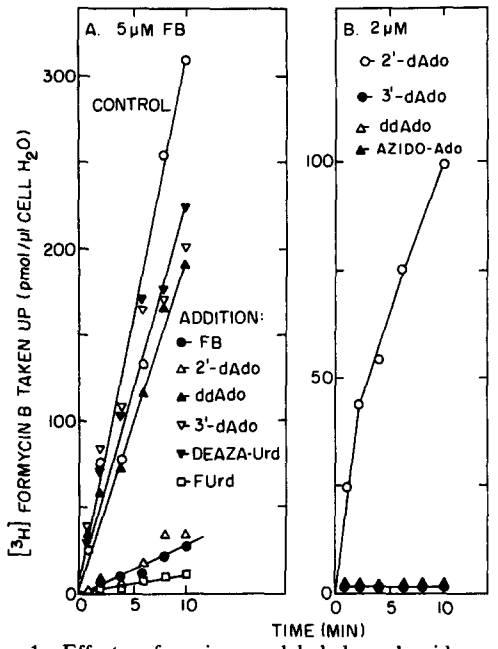

Fig. 1. Effects of various unlabeled nucleosides and nucleoside analogs on the concentrative uptake of [^{3}H]formycin B by primary rat macrophage cultures (A) and uptake of ^{3}H-labeled purine nucleosides by these cells (B). (A) One-day cultures of rat macrophages in 24-well plates were overlayed with 0.5 mL RPMI and then the uptake of 5 μM [^{3}H]formycin B (17 cpm/pmol) was measured at room temperature as described under Materials and Methods. Where indicated, 100 μM unlabeled formycin B (FB), 2'-deoxyadenosine (2'-dAdo) 3'-deoxyadenosine (3'-dAdo),2',3'-dideoxyadenosine (ddAdo), 6-deazauridine (Deza-Urd) or 5-fluorouridine (FUrd) was added simultaneously with the [^{3}H]formycin B. (B) The uptake of 2 μM [^{3}H]2'-deoxyadenosine (150 cpm/pmol), [^{3}H]3'-deoxyadenosine (320 cpm/pmol), [^{3}H]2',3'-dideoxyadenosine (285 cpm/pmol) and [^{3}H]azidoadenosine (Azido-Ado; 170 cpm/pmol) was measured as described for the uptake of [^{3}H]formycin B. The values for ddAdo (△—△) are obscured by those for 3'-dAdo and Azido-Ado.

RESULTS AND DISCUSSION

The substrate specificity of the Na^+-dependent, concentrative transporter of rat macrophages was investigated in two ways. In one approach we determined the effect of an excess of various unlabeled nucleosides and nucleoside analogs (all at 100 μM) on the concentrative uptake of 5 μM [^{3}H]formycin B by cultured rat macrophages (Fig. 1A) and in the other approach we directly measured the uptake of radiolabeled nucleosides (Fig. 1B). Formycin B is an efficient substrate for both the equilibrative and Na^+-dependent, concentrative transporter of mammalian cells, but it is a poor substrate for cellular metabolic enzymes

Table 1. Effects of various unlabeled natural nucleosides and their analogs (all at 100 μM) on the active transport of 5 μM [^{3}H]formycin B by rat macrophages*

Purine nucleosides		Pyrimidine nucleosides	
Unlabeled nucleoside added	Percent inhibition	Unlabeled nucleoside added	Percent inhibition
Formycin B	>90	Uridine	>90
Adenosine	>90	5-Fluorouridine	>90
2′-Deoxyadenosine	>90	Deazauridine	<30
5′-Deoxyadenosine	>90	Cytidine	<30
2-Chloroadenosine	>90	Thymidine	<30
2′-Deoxyguanosine	>90	5-Fluorodeoxyuridine	<30
Guanosine	>90	5-Bromodeoxyuridine	<30
8-Azidoadenosine	60–70	2′-Deoxycytidine	<30
Tubercidin	60–70	6-Azacytidine	<30
6-Mercaptopurine riboside	60–70	Cytosine arabinoside	<30
5′-Methylthioadenosine	~50	2′, 3′-Dideoxycytidine	<30
Adenosine arabinoside	~50		
3′-Deoxyadenosine	<30		
2′, 3′-Dideoxyadenosine	<30		
Hypoxanthine	<30		

* The experiments were conducted as described in the legend to Fig. 1.

so that its uptake by cells can be measured unimpeded by metabolic conversions [5, 9–11, 19]. As is apparent in Fig. 1A, the rat macrophages accumulated 5 μM formycin B to about sixty times the extracellular concentration in 10 min of incubation at 25°. Previous studies have documented that the concentrative uptake is completely dependent on Na^+, is abolished by treatment of the cells with the Na^+/K^+ ionophore gramicidin, and is little or not affected by NBTI, dipyridamole and other inhibitors of equilibrative nucleoside transport and that the K_m values for Na^+ and formycin B co-transport are about 10 mM and 5 μM, respectively [11]. The uptake of radioactivity from 5 μM [^{3}H]formycin B was reduced >90% by the presence of 100 μM unlabeled formycin B (Fig. 1A), reflecting the saturation of the transporter [11].

The effects of various other unlabeled nucleosides on formycin B transport were assessed as described for unlabeled formycin B and as illustrated in Fig. 1A. The overall results derived from at least two independent analyses for each nucleoside are summarized in Table 1. Three levels of inhibition were observed: (1) >90%, just as observed for 100 μM unlabeled formycin B (see Fig. 1A). Nucleosides exhibiting this degree of inhibition are considered primary substrates for the concentrative nucleoside transporter. The nucleosides falling in this group included all natural purine ribo- and deoxyribonucleosides as well as 2-chloroadenosine and of the pyrimydine nucleosides only uridine and 5-fluorouridine. (2) 50–70%. Nucleosides exhibiting this degree of inhibition are considered to possess

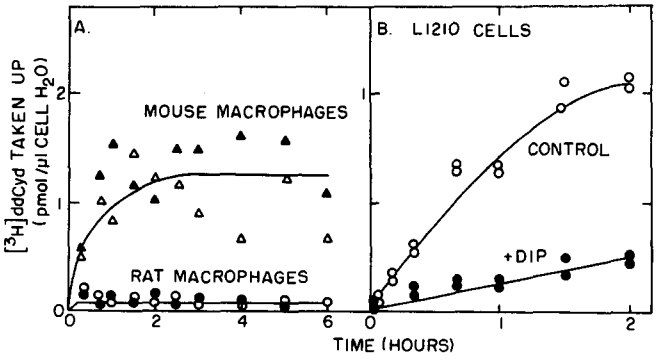

Fig. 2. Dideoxycytidine uptake by rat and mouse macrophages (A) and L1210 cells (B) and the effect of dipyridamole. (A) Two-day cultures of rat and mouse macrophages in 24-well culture plates were overlayed with 0.5 mL RPMI and then the uptake of 1 μM [^{3}H]dideoxycytidine (200 cpm/pmol) was measured as described under Materials and Methods. Where indicated, dipyridamole was added to the wells to 10 μM before addition of the labelled substrate. (B) Two samples of a suspension of 8×10^7 L1210 cells/mL of RPMI were supplemented with 1 μM [^{3}H]dideoxycytidine (100 cpm/pmol) and one sample also with 10 μM dipyridamole about 2 min before addition of the labeled substrate. At the indicated times of incubation at 25°, the cells from 0.5 mL of suspension were collected by centrifugation through an oil layer and analyzed for radioactivity. Symbols in A: (○—○, ●—●) rat macrophages, and (△—△, ▲—▲) mouse macrophages without and with dipyridamole, respectively.

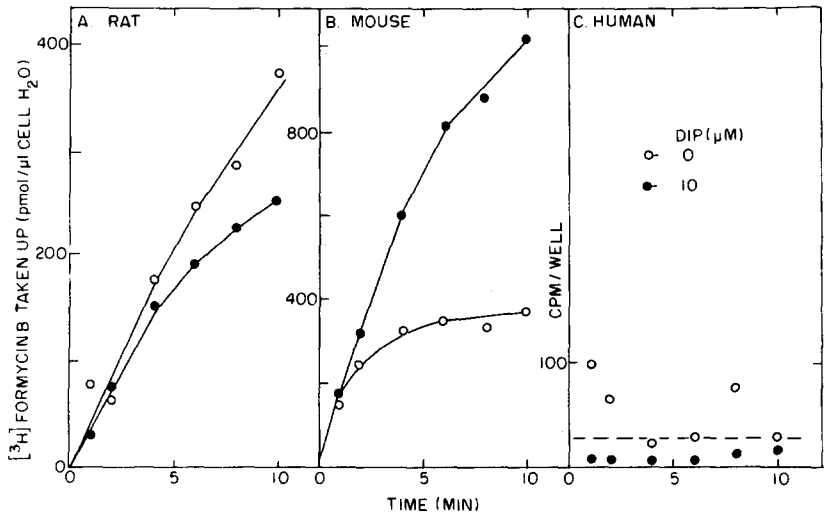

Fig. 3. Uptake of formycin B by rat (A), mouse (B), and human (C) macrophages and the effect of dipyridamole. Cultures of macrophages in 24-well culture plates were overlayed with 0.5 mL RPMI and then the uptake of 5 µM [^{3}H]formycin B (100 cpm/pmol in A and 140 cpm/pmol in B) was measured as described under Materials and Methods. Where indicated, dipyridamole was added to the wells to 10 µM before addition of the labeled formycin B.

only moderate affinity for the concentrative transporter. Whether they are transported by this carrier, however, cannot be decided on the basis of these data. This group was composed of a number of purine nucleoside analogs. (3) <30%. Because of some variability in the uptake curves due to the low density of these macrophage cultures, this degree of inhibition was not significant and the nucleosides falling in this group therefore are not considered to be substrates for the concentrative nucleoside transporter. This group included all other pyrimidine nucleosides tested, including 2′,3′-dideoxycytidine, as well as 3′-deoxyadenosine and 2′,3′-dideoxyadenosine. The lack of substrate activity of the latter purine nucleosides was confirmed by direct uptake measurements (Fig. 1B). No significant accumulation of 3′-deoxyadenosine or 2′,3′-didoxyadenosine was observed, whereas 2′-deoxyadenosine accumulated intracellularly to fifty times the extracellular concentration in 10 min of incubation. Lack of uptake of 3′-deoxyadenosine and 2′,3′-dideoxyadenosine was not due to rapid deamination since deamination was prevented completely by the presence of deoxycoformycin (data not shown). The results indicate that the concentrative nucleoside transporter resembles the equilibrative nucleoside transporter [2, 20] in expressing low or no affinity for nucleosides that lack a 3′-OH group. Overall, the substrate specificity of the concentrative transporter is much more restricted than that of the equilibrative transporter in that it fails to transport any pyrimidine nucleoside other than uridine (and 5′-fluorouridine).

Azidoadenosine was of special interest, because of its potential as a photoaffinity label for the concentrative nucleoside transporter. However, our results indicate that it is not a substrate for this transporter, even though it moderately inhibited the concentrative transport of formycin B by the rat macrophages (Table 1); no significant uptake of [^{3}H] azidoadenosine by rat macrophages could be detected (Fig. 1B). On the other hand, 8-azidoadenosine is an efficient substrate for the equilibrative transporter of mammalian cells [2].

2′,3′-Dideoxycytidine was also of special interest since it is an inhibitor of HIV replication in human macrophage/monocyte cultures and dipyridamole has been found to potentiate its effectiveness in inhibiting HIV replication in these cultures [14, 21]. The mechanism of this effect of dipyridamole has not been explored. It could have been mediated in the same manner as the enhancement of deoxyadenosine toxicity by dipyridamole for mouse L1210 leukemia cells [13], namely through enhancing the concentrative accumulation of the toxic nucleoside by the Na^+-dependent, active transporter by inhibiting its efflux via the equilibrative transporter [10, 11]. Such a mechanism, however, would require that the human macrophages/monocytes express both equilibrative and concentrative transport and that dideoxycytidine is a substrate for both transporters. These requirements are not fulfilled. Dideoxycytidine is a substrate for the equilibrative nucleoside transporter of certain mammalian cells, but it is transported only 1% as rapidly as natural substrates, such as uridine or adenosine [22]. Furthermore, as indicated by the data in Table 1, it is not a substrate for the Na^+-dependent, concentrative carrier of rat macrophages. Lack of substrate activity of dideoxycytidine for the concentrative carrier was substantiated by measuring the direct uptake of [^{3}H]dideoxcytidine by rat and mouse macrophages and L1210 cells (Fig. 2). [^{3}H]Dideoxycytidine was not taken up to a significant

extent by rat macrophages, which express only the concentrative nucleoside transporter (Fig. 2A). It slowly equilibrated with the intracellular space of mouse macrophages and L1210 cells, which express both equilibrative and concentrative nucleoside transport, but dipyridamole did not enhance its accumulation (Fig. 2 A and B). On the contrary, in agreement with an earlier study [22] dipyridamole inhibited [^{3}H]dideoxycytidine uptake in L1210 cells, indicating that dideoxycytidine entered these cells by equilibrative nucleoside transport. Dipyridamole had no significant effect on [^{3}H]dideoxycytidine uptake by mouse macrophages (Fig. 2A) or normal rat kidney cells (data not shown), which suggests that dideoxycytidine entered these cells mainly by non-mediated diffusion, as also reported for azathymidine and dideoxyadenosine [23, 24].

In addition we have found that human monocytes/macrophages probably lack a Na^+-dependent, concentrative nucleoside transport system; no significant uptake of [^{3}H]formycin B above equilibrium level was observed in cultures of these cells in either the presence or absence of dipyridamole (Fig. 3C). Comparable results were obtained in a second set of cultures of human monocytes/macrophages. These results contrasted with the concentrative uptake of formycin B by rat and mouse macrophages. Concentrative uptake was inhibited only slightly by dipyridamole in rat macrophages (Fig. 3A), because they express only the concentrative nucleoside transporter, but enhanced markedly in macrophages (Fig. 3B) because of the additional operation of the equilibrative nucleoside transporter. It is possible, but unlikely, that the lack of concentrative nucleoside transport in human monocytes/macrophages reflects their peripheral blood origin which differs from that of the peritoneal rat and mouse macrophages. Four human cell lines analyzed (HeLa, CEM, H-9 and Jurkat) and one monkey cell line (MA-104) all exhibit high levels of equilibrative but also no concentrative nucleoside transport, whereas all mouse cell lines and some rat cell lines express both [10, 11]. In any case, the human monocyte/macrophage cultures in which dipyridamole has been reported to potentiate the inhibition of HIV replication by dideoxycytidine were similarly established with peripheral blood monocyte/macrophages [14]. Thus our results suggest that dipyridamole does not potentiate the dideoxycytidine inhibition by inhibiting the efflux of dideoxycytidine that is actively transported into the cells. First, dideoxycytidine is not a substrate for the active nucleoside transporter and only a very poor substrate for the equilibrative nucleoside transporter [22] and, second, human monocytes/macrophages seem to lack an active nucleoside transporter.

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