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THE SALVAGE OF DEOXYCYTIDINE INTO dCDP-DIACYLGLYCEROL BY MACROPHAGES AND LYMPHOCYTES

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Summary: Extracellular deoxycytidine(CdR) was previously shown to be salvaged into water soluble[1] and also into lipidic [2] precursors of phospholipids in stimulated lymphocytes and in lymphoma cells[3]. In this paper we have described that non-dividing murine macrophages salvaged not only 5-^{3}H-CdR but also tritiated thymidine(^{3}H-TdR) mainly into the pools as nucleotides. Chlorpromazine shifted the CdR salvage into a lipidic compound of the cells which was identified as ^{3}H-dCDP-diacylglycerol(dCDP-DAG). After 5-^{3}H-CdR labeling the lipid/DNA ratio was eleven times higher in macrophages than in tonsillar lymphocytes. Thin layer chromatography(TLC) on borate impregnated silica gel plates gave clear separation of CDP-DAG from dCDP-DAG supporting that the extracellular precursor for it is exclusively deoxycytidine and not ribocytidine. No interconversion between deoxy- and ribocytidine could be observed neither in lymphocytes nor in macrophages. © 1993 Academic Press, Inc.

While de novo biosynthesis of nucleotides usually paralells the duplication of DNA, salvage of nucleosides gave unambiguous results. Extracellular thymidine incorporation follows the DNA replicating cycle of the cells, while the phosphorylation of CdR did not follow it[4] although it was thought to be utilized only for DNA. We found also remarkable differences between lymphoid organs with respect of phosphorylation of CdR and TdR[5] and a preferential utilization of CdR compared to TdR was found in undifferentiated(peanut positive) follicular cells of tonsils[6]. The regulation of thymocyte proliferation by deoxycytidine, excreted by follicular dendritic cells has been shown[7], but the mechanism of this effect is still not clear. Many anticancer, antiviral and anti-inflammatory drugs were activated via the salvage pathway of CdR; attention was focused on its metabolism, mainly in lymphoid cells. It turned out that CdR is salvaged by cells not only into DNA but also into water-soluble phospholipid precursors (dCDP-choline and dCDP-ethanolamine) in fibroblasts[8] and even more in tonsillar lymphocytes[1]. In the presence of chlorpromazine the lipidic

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dCDP-DAG was also detected in sarcoma cells [9] and in lymphocytes[10]. Before the in vivo experiments by intact cells little attention was drawn to observations in homogenates where Kennedy could activate phospholipid precursors both by CTP and dCTP[11].

Mononuclear phagocytes play a central role in chronic inflammatory diseases. Blood monocytes do not synthesize deoxynucleotides de novo and their transformation into macrophages occurs without cell division. Chihiro et al.[12] have shown that monocytes rapidly repair DNA strand breaks induced by gamma-irradiation. Compared to the most other cell types it has been shown, monocytes and macrophages have very low dCTP pools but abundant deoxycytidine kinase activity[12].

In the present work the salvage of CdR and TdR were investigated in macrophages which have no DNA synthesis [13,14] but have active DNA repair and membrane-bound processes(e.g. phagocytosis); comparisons were made also in activated lymphocytes, which have DNA synthesis.

MATERIALS AND METHODS

Isolation of tonsillar lymphocytes from surgically removed tonsils of 3-6 years old children and murine peritoneal macrophages were performed according to our previous studies[15,16]. Briefly, 5 x 10^6 lymphocytes or 5 x 10^6 macrophages were cultured in 1 ml media and labeled with 5-^{3}H-CdR(Amersham, sp. act. 740 GBq/mmole), with 1'-2'-5-^{3}H-CdR(Amersham, sp. act. 1890 GBq /mmole) and with 5-^{3}H-TdR(UVVVR, Prague, sp. act. 888 GBq/mmole), respectively at 37°C for indicated times. After incubation at 37°C, cells were cooled to 0°C, washed and precipitated by 70% ethanol and kept overnight at - 20°C. Aliquots of the cold ethanol-soluble fraction were measured for total (pool) radioactivity and DEAE-cellulose paper bound radioactivity (nucleotides) [17]. Liponucleotides were calculated as the difference between the total and DEAE-bound radioactivity.Incorporation into DNA was the radioactivity measured in the hot $HClO_4$ hydrolysate of the insoluble precipitate [2,17].

Lipids were extracted from the acid insoluble fraction of cells as described[2]: by 0.5 ml chloroform:methanol(1:1) with 0.1 M HCl. Acid soluble fraction was considered as the total pool while incorporation into DNA was measured in the acid hydrolysate of the acid-insoluble fraction.

Thin layer chromatography was performed on 0.4 M boric acid impregnated silica gel plates(Kieselgel 60 F254, Eastman-Kodak). 60 µl Samples of lipidic extracts were chromatographed in an eluant containing a mixture of chloroform:methanol:water:ammonia (35:19:1:4) for 5 hrs[9]. After drying the sheets were cut in 2 x 1 cm slices and measured for radioactivity. CDP-DAG(Sigma) was used as marker. Picomoles were calculated from the specific radio-activity of the extracellular precursor.

RESULTS AND DISCUSSION

Macrophages salvage the two pyrimidine deoxynucleosides mainly into the pool with the same efficacy

Table I shows the salvage of the two deoxypyrimidine nucleosides in murine macrophages and human tonsillar lymphocytes. As it can be seen the total uptake(pool+DNA) of both labeled nucleosides was much higher in lymphocytes than in macrophages. While about half of labeled CdR can

Table 1. Comparison of the salvage of CdR and TdR in lymphocytes and macrophages

Labeling	cpm (pmol) per 10^6 cells				
Cells	uptake	pool	nucleotide	LN	DNA
5-^{3}H-CdR					
Lymphocytes	8040	3999	1595	2505	4040
	(0.398)	(0.198)	(0.08)	(0.125)	(0.20)
Macrophages	1520	920	720	230	600
	(0.075)	(0.045)	(0.035)	(0.011)	(0.03)
5-^{3}H-TdR					
Lymphocytes	22375	375	300	60	22000
	(0.96)	(0.016)	(0.013)	(0.002)	(0.95)
Macrophages	1830	950	460	530	880
	(0.079)	(0.041)	(0.019)	(0.023)	(0.038)

Picomoles were calculated on the basis of the specific activities of the labeled nucleosides considering the efficacy of the measurement of radioactivity. Uptake: the total radioactivity in the cells; pool: the radioactivity in the 70 % ethanol soluble fraction; nucleotide: radioactivity bound to DEAE-cellulose; LN(liponucleotide): pool - nucleotides; DNA: labeling precipitable by 70 % cold ethanol.

be found in the acid soluble pool both in lymphocytes and macrophages, only 1.8 % of labeled TdR remains in the pool in lymphocytes and 52 % in macrophages. The phosphorylated part of the labeled pools was also determined by binding on DEAE cellulose sheets[17] and it refers labeled nucleotides (NMP, NDP, NTP). The most striking observation from Table I is that macrophages salvage roughly the same amount of CdR in the nucleotide fraction as TdR while lymphocytes have several times more labeled CdR in the pool than TdR and the main part of TdR is incorporated into DNA. The higher activity of the pool after ^{3}H-TdR labeling in macrophages can be the consequence of the absence of DNA synthesis and of the pronounced catabolism of TdR[18], which is in agreement with the earlier observations of Stadecker et al.[13,14] that macrophages produce and secrete thymidine.

Lipidic dCDP-DAG was accumulated in the presence of chlorpromazine

Chlorpromazine, an amphiphilic amine was found to stabilize the lipidic nucleotide derivatives of CdR[19]. Figure 1 shows the effect of chlorpromazine on the CdR metabolism. As it can be seen 20 μM of the drug decreases the CdR label in the pool and increases it continuously

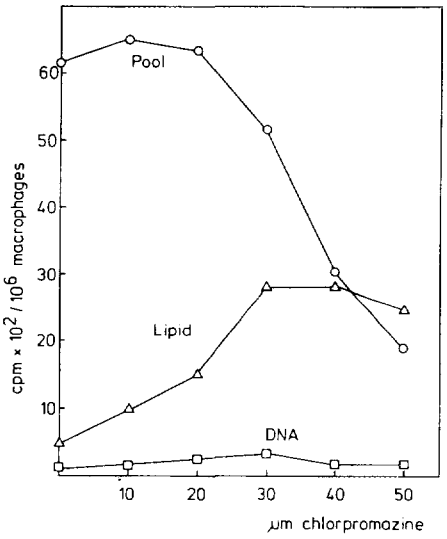

Fig. 1. The effect of chlorpromazine on CdR metabolism in murine peritoneal macrophages
5 x 10^6/ml peritoneal macrophages were labeled by 200 kBq 5-^{3}H-CdR in the presence of chlorpromazine at the indicated concentrations. Symbols are given in the Figure. Pool contains nucleotides and liponucleotides. Lipid fraction: the chloroform/methanol/0.1 N HCl extract of acid-insoluble fraction. DNA fraction contains the labeling of the acid-insoluble fraction after lipid extraction.

in the lipidic fraction of the macrophages. The acid precipitable ^{3}H-CdR(DNA) was low and shows slight change as a consequence of chlorpromazine treatment. Lymphocytes were more sensitive against to this treatment; the labeling of the pool and the DNA decreased more drastically[2] than in macrophages. The lipidic extracts of chlorpromazine-treated and untreated cells were analysed on borate impregnated silica plates. A fairly good separation of ribo- and deoxyribonucleotide derivatives could be achieved. Cells were labeled by 5-^{3}H-CdR for one hour and extracted for lipid and separated on TLC. As it can be seen some(20 %) labeled CDP-DAG and dCDP-DAG were obtained without chlorpromazine but 80 % of the radioactivity appears as dCDP-DAG when the cells were treated with chlorpromazine. The enhancement of dCDP-DAG formation from extracellular 5-^{3}H-CdR was explained by the inhibition of phosphatidate phosphohydrolase by chlorpromazine observed in vitro[19], dropped its turnover. Similar enhancement was observed by chlorpromazine in peripheral blood lymphocytes and sarcoma cells[9,10]. One can also see in Fig. 2 that chlorpromazine increased at least four times the labeling of dCDP-DAG while during the same time the labeling of CDP-DAG decreased by the same extent. This observation invites speculations of a possible separate pathway of CdR into ribo- and deoxyribo-nucleotide phospholipid precursor pools while only the latter

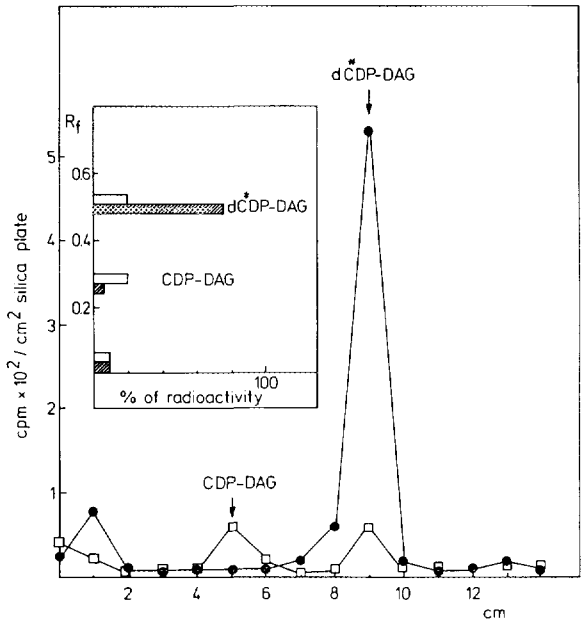

Fig. 2. TLC of macrophage lipid extracts labeled by 5-^{3}H-CdR in the presence (• - •) and absence (□ - □) of chlorpromazine

60 µl lipidic extracts were chromatographed on 0.4 M borate-impregnated silica plates using an eluant containing chloroform-methanol-water-ammonia(35:19:1:4) for 5 hrs. Nucleotides were visualized under uv. light. Data on the abscissa show the length of the cut slices from the start. Slices were measured for radioactivity.

was enhanced by the membrane active drug chlor-promazine. The exact mechanism of the amphiphilic amine (widely used as sedative drug in the psychyatry) on the transport and metabolism of nucleosides is not clear yet; its effect on monovalent cation transport has to be taken into consideration.

It has to be emphasized that macrophages are less sensitive to higher chlorpromazine concentrations(Fig. 1) than activated lymphocytes[2]. where the optimal concentration range is very narrow for stimulating the incorporation into lipids. The ratio of 5-^{3}H-CdR incorporation into lipids and into DNA was about eleven times higher in macrophages than in lymphocytes referring to a very active membrane biosynthesis i.e. to an active anionic phospholipid biosynthesis(phosphatidyl-inositols and glycerols) and to a low DNA synthesis.

Deoxycytidine salvage is not connected to ribocytidine salvage neither in lymphocytes nor in macrophages

Previously we showed in lymphocytes using differently labeled CdR that there is no conversion of deoxyribocytidine into ribocytidine[2]. On the other hand a hundredfold excess of ribocytidine to CdR did not decrease the labeling from CdR[1,2]. Figure 3 shows that in macrophages

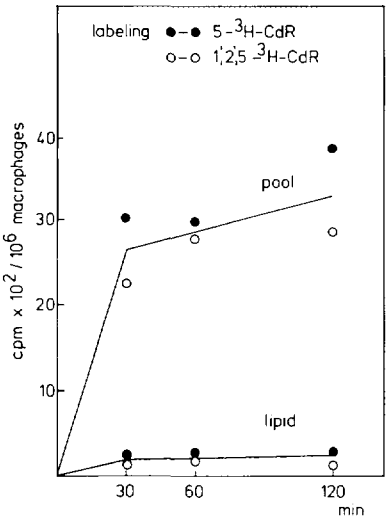

Fig. 3. Metabolism of the pyrimidine and sugar labeled CdR in murine peritoneal macrophages

5 x 10^6/ ml peritoneal macrophages were labeled by 200 kBq 5-^{3}H-CdR and 1',2',5-^{3}H-CdR. Samples were treated and measured according to Fig. 1.

when cells incorporated CdR labeled either in the pyrimidine ring(5-^{3}H-CdR) or also in the sugar part(1',2',5-^{3}H-CdR) at the same amount of total radioactivity, no significant difference was obtained in the labeling of the pool and of the lipidic fraction. If the cells would be able to remove deoxyribose from CdR, the radioactivity of the pool (nucleotides+liponucleotides) and also of the lipids should be lower from sugar labeled CdR. The results suggest the lack of enzyme(s) converting deoxyribonucleosides to their ribo-counterpart. The salvage of 5-^{3}H-CdR into water-soluble[1] and into lipidic[2] phospholipid precursors has been shown by different methods in human lymph node lymphocytes. It had to be further proved by new methods that pyrimidine ribo- and deoxyribonucleosides are salvaged separately and are not interconverted into each other.

For the unambiguous separation of ribo- and deoxyribo-pyrimidine derivatives the borate impregnated silica gel TLC was adopted[9]. Lymphocytes were labeled by 5-^{3}H-CR, 5-^{3}H-CdR and 1',2',-5-^{3}H-CdR in the presence of 30 μM chlorpromazine. The lipidic extracts were separated on borate impregnated TLC(Fig. 4). Some (20 %) of the radioactivity remains at the start line from all three labeled precursors, the nature of that lipidic compound is still unknown. If the cells were labeled by the ribonucleoside (5-^{3}H-CR) 60 % of the labeling appears at the position of CDP-DAG, co-cromatographed with the lipidic extract, only 6 % appears at position of dCDP-DAG. This small part of salvaged CR has to

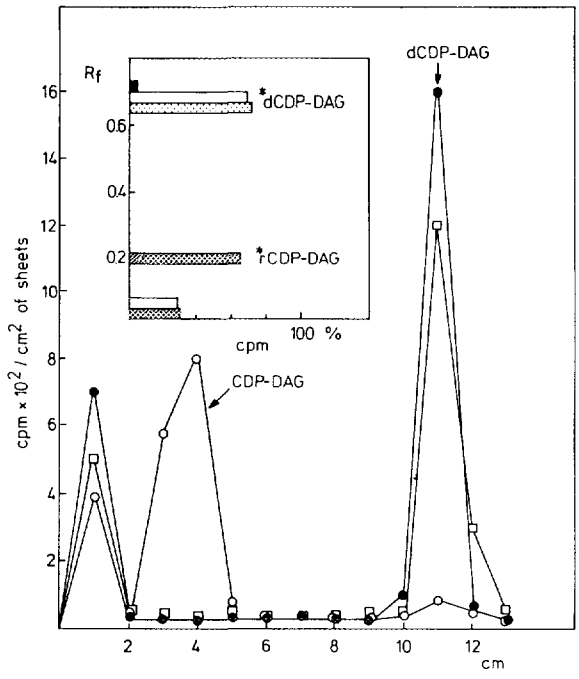

Fig. 4. TLC of lymphocyte lipid extracts labeled by 5-^{3}H-CR (o - o), 5-^{3}H-CdR (• - •) and 1', 2',-5-^{3}H-CdR(□ - □)
Chromatography was performed according to Fig. 2.

be mixed with the de novo CTP pool and reduced via ribonucleotide reductase to dCTP [20].

If the lymphocytes were labeled either in pyrimidine ring or in sugar CdR only one well defined labeled spot appeared with a R_f value 0.68 and defined as dCDP-DAG(Fig. 4). This experiment strongly supports that deoxyribocytidine will not be converted into its ribo-counterpart, has its own specific way in the phospholipid activation process. The result is in good agreement with the earlier findings of Perignon et al.[21], who found that human lymphocytes lack the capacity of nucleotide synthesis from pyrimidine bases. It would be important to know what can be the specific function of deoxycytidine in the phospholipid synthesis. While lymphocytes contain 23 pmole CTP and about 1-3 pmole dCTP per 10^6 cells[22], they still salvage deoxycytidine intensively into the phospholipid membrane dCTP pool as shown earlier[22]. We have suggested that the membrane dCTP pool has connections to DNA repair which can be inhibited by dATP. However, any other kind of "non-S-phase" DNA synthesis(i.e. during cell differentiation) may also use the membrane dCTP pool[23,24]. Studies on the function of the extracellular deoxycytidine in connection with the membrane and DNA repair processes

are in progress both with macrophages and lymphocytes. One possible function of the pronounced deoxycytidine salvage in lymphoid and mononuclear cells can be to maintain the concentration of CdR at a constant level in the blood similarly to thymidine[25], which may occur by a continuous uptake and excretion of the nucleosides[20].

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