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Effects of dipyridamole on human blood lymphocytes*

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Dipyridamole [2,6-bis(diethanolamino)-4,8-dipiperidino (5,4-d)pyrimidine] inhibits the transport of purine and pyrimidine nucleosides through the membranes of normal and malignant mammalian cells [1]. This effect of dipyridamole was demonstrated in human erythrocytes [2], platelets [3] and lymphocytes [4], pig vascular endothelium [5], chicken fibroblasts [6], heart [7], murine leukemic lymphoblasts [8] and rat hepatoma cells [9]. Concentrations of dipyridamole inhibiting the influx of nucleosides into cells were lower than those altering the output [8].

We have examined the effects of dipyridamole on the uptake of [^{3}H]thymidine into human blood lymphocytes, on the stimulation of lymphocytes by PHA and on the formation of rosettes with sheep red blood cells (SRBC).

Lymphocyte suspensions. Human blood lymphocytes from healthy volunteers were isolated on a Ficoll–Hypaque gradient. Macrophages were removed by previous 30 min stirring of heparinized blood with carbonyl iron (2 mg/ml, Calbiochem AG, Lucerne, Switzerland). Lymphocytes were washed three times and suspended in Parker's medium. Platelet contamination was less than one platelet per nucleated cell.

[^{3}H]*Thymidine uptake by lymphocytes.* Lymphocyte suspensions (200 μl, containing 2.5 × 10^6 cells) were incubated with 200 μl of Parker's medium alone or containing dipyridamole (Pharma Research Canada Ltd., Quebec) and 0.2 μCi (10 μl) of [^{3}H]thymidine (2 Ci/mole, UVVV Prague, Czechoslovakia) at 37°. At the time intervals indicated, lymphocytes were spun down by centrifugation at about 700 *g* for 5 min and washed three times with Parker's medium. The cell pellets were solubilized in 0.5 ml of Nuclear Chicago Solubilizer (Amersham/Searle, U.S.A.) during overnight incubation at 37°. Radioactivity of cell lysates was measured by liquid scintillation spectrometry [10].

Incorporation of [^{3}H]*thymidine and* [^{14}C]*leucine into lymphocyte cultures.* Lymphocyte suspensions (200 μl, containing 2.5 × 16^6 cells) in Parker's medium supplemented with horse serum (15% v/v), glutamine (3% w/v) and canamycin (3 μg/ml) were cultivated using a microculture system in a humidified atmosphere of 95% air and 5% CO_2 in an ASSAB incubator at 37° for 72 hr. Dipyridamole in Parker's medium (20 μl) and 1 μl of PHA (Difco, Detroit, MI) were added before cultivation. [^{3}H]Thymidine (0.2 μCi/10 μl) or [^{14}C]leucine (0.1 μCi–62 mCi/mmole, Amersham/Searle, U.S.A.) in 20 μl was added 18 hr or 4 hr before cell harvesting, respectively. Incorporation of [^{3}H]- or [^{14}C]-radioactivity into acid-insoluble material was determined by liquid scintillation spectrometry [10].

Rosette tests. Rosettes formed during incubation of lymphocytes with sheep red blood cells (SRBC) at 4° for 1 hr were assayed according to Jondal *et al.* [11] and called E-late. The technique of Wybran and Fudenberg [12] was applied for examination of rosettes E-early formed immediately after mixing lymphocytes with SRBC at room temperature. Rosettes EA formed by Fc-receptor bearing cells were investigated as described by Benwich *et al.* [13] using SRBC sensitized with the maximal subagglutinating dose of anti-SRBC rabbit antibody (Biomed, Kraków, Poland). Dipyridamole dissolved in Hank's solution, pH 6.8, was added in a volume not exceeding 20 per cent of the total volume of the samples tested. Percentages of rosettes were calculated after counting one thousand lymphocytes.

Binding of [^{14}C]*dipyridamole to lymphocytes.* Lymphocyte suspensions (200 μl, containing 5–10 × 10^6 cells) in Hank's solution, pH 6.8, were incubated with 200 μl of [^{14}C]dipyridamole (7.3 μCi/mg, Pharma Research Canada Ltd., Quebec, Canada) dissolved in the same solution.

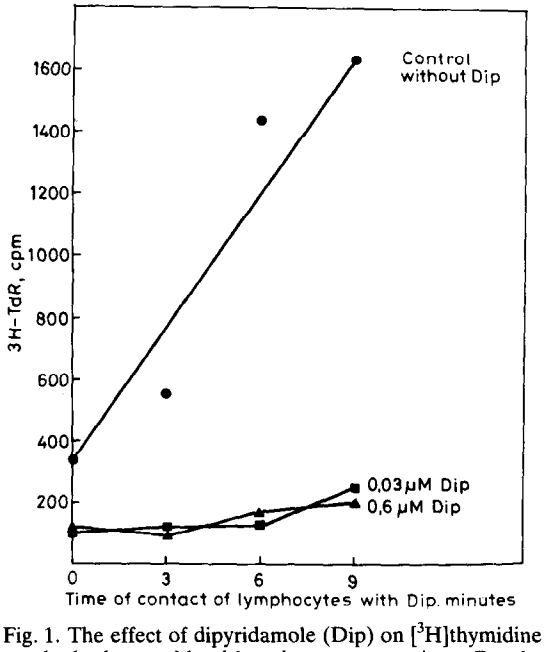

Fig. 1. The effect of dipyridamole (Dip) on [^{3}H]thymidine uptake by human blood lymphocyte suspensions. Results are mean values from determinations in four to five samples. For details see Materials and Methods.

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Table 1. The effect of dipyridamole on incorporation of [^{3}H]thymidine and [^{14}C]leucine into lymphocyte cultures. Results are expressed as mean values from determinations in four to five cultures

Dipyridamole (μM)	[^{3}H]Thymidine incorporation				[^{14}C]Leucine incorporation	
	Non-stimulated		PHA-stimulated		Non-stimulated	PHA-stimulated
	cpm	% inhibition	cpm	% inhibition	% inhibition	% inhibition
0.0	1480	—	40,296	—	—	—
0.025	1270	30.5	36,025	10.6	0	0
0.05	1005	45.5	19,987	50.4	0	0
0.5	567	62.9	4070	89.9	0	0
1.5	379	79.4	1571	96.1	16	0

After centrifugation at about 700 *g* for 5 min, supernatant fractions were collected and cell pellets washed twice with cold phosphate-buffered saline, pH 7.2. Radioactivity was measured separately in supernatant fractions and in cell pellets, solubilized as described above. The recovery of the total [^{14}C]dipyridamole added ranged from 75 to 89 per cent.

Figure 1 shows an abrupt inhibition of [^{3}H]thymidine uptake into lymphocyte suspensions by dipyridamole. Nearly complete inhibition was attained with 0.03 μM dipyridamole at zero time when lymphocytes were sedimented by centrifugation immediately after mixing with the dipyridamole solution. In the control samples, without dipyridamole, [^{3}H]thymidine radioactivity taken up by lymphocytes increased progressively over time.

The data presented in Table 1 show the dipyridamole concentration-dependent inhibition of [^{3}H]thymidine incorporation into the acid-insoluble fraction of lymphocytes cultivated for 72 hr. The effects of dipyridamole were similar in non-stimulated and PHA-stimulated cultures. About 50 and over 80 per cent inhibition was observed at 0.05 and 1.5 μM concentrations of dipyridamole, respectively. It was found that a short contact of lymphocytes with dipyridamole was sufficient to provoke alterations which could not be reversed during 72 hr cultivation. Incubation of lymphocyte suspensions with dipyridamole for 5 or 30 min, followed by washing out the drug, inhibited [^{3}H]thymidine incorporation into acid-insoluble material to the same extent as the constant presence of dipyridamole during the whole cultivation period (not shown).

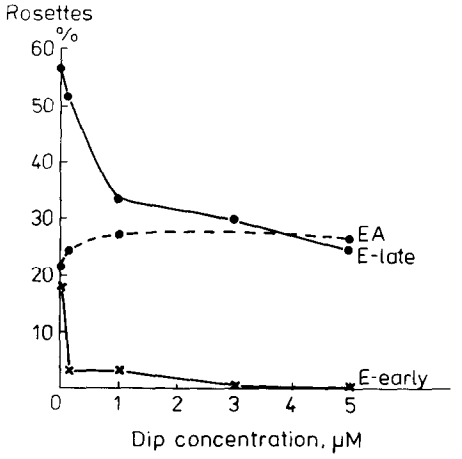

Fig. 2. The effect of dipyridamole (Dip) on formation of rosettes with sheep red blood cells (SRBC). E-early-rosettes formed immediately after mixing lymphocytes with SRBC at room temperature. E-late-rosettes formed with SRBC after 1 hr incubation at 4°. EA-rosettes with SRBC sensitized with anti-SRBC antibody.

Transformation of lymphocytes by PHA appeared to be unaffected by the applied concentrations of dipyridamole. This was evidenced by unaltered stimulation of [^{14}C]leucine incorporation into cell proteins (Table 1), agglutination and blastic transformation evaluated by morphological criteria.

The effects of dipyridamole on the ability of lymphocytes to form rosettes with SRBC are presented in Fig. 2. Low dipyridamole concentrations diminished the percentage of rosettes (E-late) formed by lymphocytes during incubation with SRBC at +4° for 1 hr. The formation of the so called 'active' or 'early' rosettes (E-early) formed during a very short contact of lymphocytes with SRBC at room temperature seemed to be most susceptible to the drug. The percentage of the E-early rosettes decreased from the control value of 18 per cent to 3 and 0 per cent at 0.1 and 1 μM dipyridamole concentrations, respectively. Dipyridamole concentrations up to 5 μM did not significantly alter the number of rosettes EA with SRBC sensitized with the maximal subagglutinating dose of anti-SRBC antibody.

According to present views, rosette E-assay detects the whole population of T lymphocytes while Fc-receptor-bearing B cells form rosettes EA. The population of blood T lymphocytes is well known to be heterogeneous in respect to functional properties. Its heterogeneity is also expressed in the affinity for SRBC. A high yield of rosette E was obtained after overnight incubation of lymphocytes with SRBC at a low temperature. The percentages of rosettes E-early formed during short contact of lymphocytes with SRBC at room temperature were much lower. The cells forming this type of rosette may represent a distinct subpopulation of T lymphocytes or their particular state may be characterized by high affinity for SRBC and preferential sensitivity to surface alterations provoked by dipyridamole. It is worth mentioning that the recently-published data point to a relation between the affinity of human T lymphocytes for SRBC and such a functional property as antibody-dependent cytotoxicity [14].

In the last experiment the binding of [^{14}C]dipyridamole to lymphocytes in suspension was examined. Figure 3 shows that on a double logarithmic scale the amount of lymphocyte-bound dipyridamole is linearly related to the drug concentration up to 2.5 μM. We have found that 2.5 μM concentration constitutes a limit for complete [^{14}C]dipyridamole solubility under the conditions applied. Scatchard plots of the binding data were not linear, indicating the presence of more than one type of dipyridamole binding sites on the lymphocyte surface [15].

In conclusion, we have shown that in *in vitro* conditions dipyridamole applied in low therapeutic concentrations [16] inhibits [^{3}H]thymidine incorporation into non-stimulated and PHA-stimulated human lymphocytes and alters their receptors for SRBC. These effects of dipyridamole most probably depend on its fast and durable binding to lymphocytes. This mechanism was found to underlie the dipyr-

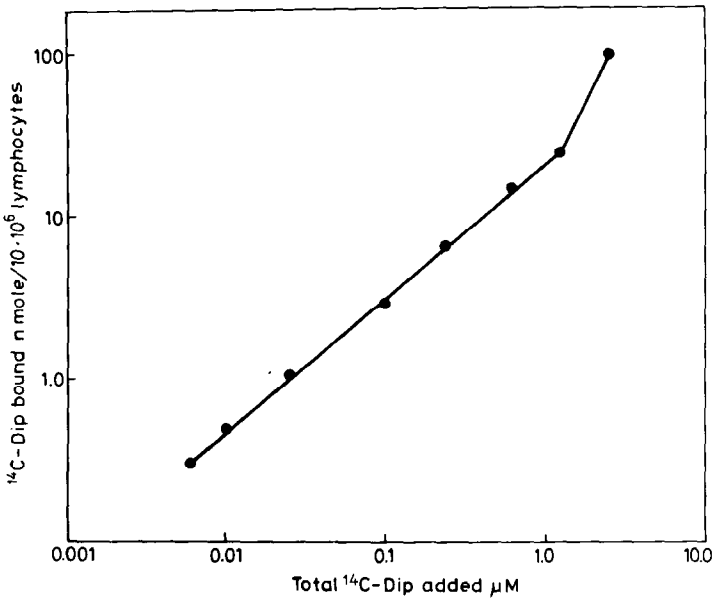

Fig. 3. Binding of [^{14}C]dipyridamole ([^{14}C]Dip) to lymphocytes. Results are means from determinations in two to three samples. For details see Materials and Methods.

idamole-provoked inhibition of adenosine binding to blood platelets as well as inhibition of their aggregation. It should be mentioned that close similarity of lymphocyte and platelet surface proteins accessible to iodination by lactoperoxidase has been demonstrated by Tanner and Boxer [17].

Coeugniet [18] reported that dipyridamole impairs the release of leukocyte and thrombocyte migration inhibitor from lymphocytes stimulated with concanavalin A. Our data indicating alterations in lymphocyte surface receptors support the assumption of this author that dipyridamole can produce effects of a possible significance for lymphocyte function.

The beneficial therapeutic effects of dipyridamole in cardiovascular diseases [19, 20], kidney diseases [21] and [22] graft rejection, and in chronic aggressive hepatitis [23] were attributed to the antiplatelet and vasoactive action of the drug. The functional modification of the lymphocyte and hence, of its immunological reactivity may be considered as a contribution to the therapeutic action of dipyridamole; therefore it deserves further study.

In summary, dipyridamole binds to human blood lymphocytes and induced an abrupt, irreversible inhibition of [^{3}H]thymidine uptake and incorporation into non-stimulated and PHA-stimulated lymphocyte cultures. Dipyridamole alters the lymphocyte receptors involved in the formation of 'early' rosettes with SRBC.

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