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SMALL LYMPHOCYTE PHOSPHORYLATION OF CYTARABINE—AN ORGAN AND SPECIES SURVEY

GARY D. GRAY,* MARY M. MICKELSON, THOMAS C. HALL and DAVD KESSEL

Department of Hypersensitivity Diseases, The Upjohn Company, Kalamazoo, Mich. 49001 and The Departments of Oncology and Pharmacology, University of Rochester Medical School, Rochester, N.Y. 14620, U.S.A.

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Abstract—The small lymphocytes isolated from the thymus phosphorylated (activated) cytarabine (*ara*-C, cytosine arabinoside) to a greater extent compared with those isolated from the spleen, lymph nodes and blood of mice, rats, rabbits, dogs and rhesus monkeys. These results were consistent with a previously stated hypothesis that adamantoyl cytarabine primarily suppressed thymus-dependent immunologic responses in rats. Although each species had a fairly unique distribution of activity (except mice and rats, which were almost identical), man differed in that not only were the blood lymphocytes far more active than those of the other species, they were equal in activity to the thymocytes.

CYTARABINE† is a useful antitumor,[1–5] antiviral,[6–11] and immunosuppressive agent[12] in man. These activities are primarily due to inhibition of DNA synthesis.[13,14] After phosphorylation of cytarabine by deoxycytidine kinase[15–18] and ubiquitous nucleotide kinases, the final intracellular product, *ara*-cytidine triphosphate, is a very effective inhibitor of DNA polymerase.[19]

The current studies with cytarabine were prompted by two separate series of observations. One dealt with the selective immunosuppressive specificity of cytarabine. For instance, in man, cytarabine suppresses primary and secondary humoral antibody responses, as well as the development of delayed hypersensitivity reactions, but has no effect on established delayed hypersensitivity responses.[12] In dogs, cytarabine suppresses the humoral antibody response to sheep erythrocytes, but it does not suppress renal transplant rejection.[20,21] Further, even a more potent derivative of cytarabine, viz. adamantoyl cytarabine,[22–26] possesses immunosuppressive specificity, and it was hypothesized that this compound selectively suppresses thymus-dependent immunologic reactions in rats.[27]

The other series of observations[28–30] showed that the antitumor activity of cytarabine could be explained on a biochemical basis: a linear relationship exists between the therapeutic activity of cytarabine in mice bearing different types of tumors and the ability of those tumor cells to phosphorylate cytarabine *in vitro*.[31] There is also a correlation between the ability of cytarabine to induce clinical remission of leukemia in man and the ability of the leukemic cells to phosphorylate cytarabine.[32] In line with these observations, an inverse correlation is seen between the activity of the

* Reprint requests.

† *Ara*-cytidine, *ara*-C, cytosine arabinoside, Cytosar (The Upjohn Company), 1-β-D-arabinofuranosylcytosine.

enzyme responsible for cytarabine inactivation (cytidine deaminase) and the initial therapeutic response in leukemic patients. Furthermore, the reduced susceptibility of the leukemic cells observed in some patients after prolonged cytarabine therapy can be associated with an increase in the activity of this enzyme.[33]

Thus, the available biochemical evidence suggests that the primary event determining the sensitivity of a cell to cytarabine is the ability of that cell to phosphorylate cytarabine (which would, in turn, be a reflection of the relative intracellular activities of the kinase and the deaminase).

The current investigation was originally designed to further test the hypothesis alluded to above, i.e. it was suspected that the thymus-derived lymphocytes of rats would be the most active cytarabine phosphorylating cells compared with lymphocytes derived from the blood, the spleen, and the lymph nodes. This study then evolved into a much broader survey to determine whether the small lymphocytes of any common animal species would resemble those of man with respect to cytarabine phosphorylating capability.

EXPERIMENTAL

Source of lymphocytes. The lymphocytes were obtained from 25 g male ICR mice, 180 g female Sprague–Dawley rats, 2–3 kg male and female rhesus monkeys, 3 kg male and female New Zealand rabbits, and 10 kg male Beagle dogs. In man, lymphocytes were isolated from adult male blood and from the thymus of children aged 4–14 at the time of thoracic surgery. The lymph nodes utilized in these experiments were as follows: mouse and rat, sublingual, axillary, inguinal and mesenteric; dog, mesenteric and axillary; rabbit, sublingual, inguinal and axillary; rhesus monkey, inguinal, mesenteric and axillary. The subjects and animals used in this study were not specifically immunized.

Purification of lymphocytes. The lymphocytes were isolated by a modified Hypaque–Ficoll isopycnic centrifugation procedure[34] from cell suspensions prepared in Hank's balanced salt solution (HBSS) as described previously.[35,36] Concentrated spleen and thymus suspensions were diluted to a final ratio of 15 ml HBSS/g of tissue. Concentrated lymph node suspensions were first centrifuged and then diluted at a ratio of about 30 ml HBSS/1 ml packed cell volume. The blood samples were shaken (defibrinated) with a sufficient number of 3 mm glass beads (4 mm beads were used for rabbit blood) to cover $\frac{3}{4}$ of the bottom of the appropriate vessel. This markedly reduced platelet contamination. The blood from individual mice and rats was swirled in 10- and 50-ml Erlenmeyer flasks, respectively, on a high speed rotary shaker. The other blood samples were gently swirled in 50-ml aliquots in 100-ml serum bottles. After defibrination, the mouse blood was diluted 1:4 with HBSS, whereas the other blood samples were mixed in a ratio of 3 vol. blood to 1 vol. of a freshly prepared 3% gelatin in saline solution at 37°.[37] After standing for 40 min, the top $\frac{1}{3}$–$\frac{1}{2}$ of the mixture was removed and diluted 1:1 with HBSS. This procedure reduced the erythrocyte contamination and improved the overall yield of lymphocytes after layering. Usually, 25 ml of the suspensions was carefully layered over 10 ml of the Hypaque-Ficoll (HF) solution[34] and centrifuged at 250 *g* (measured at the bottom of the tube) for 25 min. With the exception of human blood, which was centrifuged at 250 *g*, the blood preparations were centrifuged at 500 *g* for 25 min. If the lymphocyte layer was grossly contaminated with erythrocytes, an additional 10 min of centrifugation was employed.

The lymphocytes were removed, washed twice with HBSS, once with TES and resuspended in TES at a concentration of 1×10^8 lymphocytes/ml. TES was a buffer which contained 62 mM TES [*N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid], pH 7·2; 65 mM NaCl; 15 mM KCl; and 8 mM $CaCl_2$.

The final organ preparations consisted of at least 96 per cent lymphocytes (the average was 98 per cent lymphocytes) as determined by Wright's stain. The purity of the blood preparations was less consistent, ranging as low as 89 per cent, with an average purity of 92 per cent. The main contaminants were monocytes or blast-type cells; neutrophils and eosinophils were only rarely observed. Purification of dog blood lymphocytes proved to be the most difficult (the lymphocytes were often only 70 per cent pure) and necessitated extra experiments to attain more highly purified preparations. The erythrocyte contamination was less than 5 per cent (5 erythrocytes/100 lymphocytes) in the spleen, lymph node and thymus preparations and was usually around 25 per cent in the blood preparations. The erythrocyte contamination was unimportant, however, since preliminary experiments showed that erythrocytes did not phosphorylate cytarabine, nor did they interfere in any way under the conditions described below. With the exception of two human thymus preparations (72 and 76 per cent viable), all the preparations were at least 85 per cent viable (measured by trypan blue exclusion) and the viabilities usually exceeded 90 per cent.

Phosphorylation assay. The phosphorylation assay depends on three factors: that cytarabine is essentially freely diffusable across cell membranes;[30,31] that the primary subsequent event is the deoxycytidine kinase-catalyzed phosphorylation of cytarabine at the 5′ position;[15–18] and that phosphorylated cytarabine (present as the mono, di or triphosphate) diffuses very slowly out of cells.[32] Therefore, the assay involves incubation of lymphocytes with radioactive cytarabine; the lymphocytes are then placed in a cytarabine-free medium which allows the radioactive cytarabine to diffuse out and finally, after cell lysis, the retained radioactive phosphorylated cytarabine metabolites are measured by scintillation techniques.

Radioactive cytarabine ([^{3}H])-cytosine arabinoside, labeled in the pyrimidine ring, 1·1 c/mmole (Schwartz-Mann; Orangeburg, N.Y.) was diluted with unlabeled cytarabine to approximately 10,000 counts/min/nmole. The incubation system contained 0·05 mM cytarabine and 2×10^7 lymphocytes in a total volume of 0·21 ml in TES buffer. The incubation vessels were 1-ml disposable centrifuge tubes (Fischer–Porter; Warminster, Pa.). The incubation was conducted at 37° for 15 min. The reaction was stopped by the addition of 0·5 ml ice-cold TES. The tubes were immediately centrifuged for 25 sec in a Misco model 5500 microcentrifuge (Microchemical Specialties Co., Berkeley, Calif.). The supernatants were discarded and the lymphocytes were resuspended in 0·5 ml ice-cold TES. The suspension was reincubated at 37° for an additional 5 min to allow diffusion of cytarabine out of the lymphocytes. The suspensions were centrifuged as above, the supernatants were discarded and the cells were resuspended in 0·25 ml saline. Aliquots (0·2 ml) were placed in BBS-3 scintillation solution. The BBS-3 scintillation solution was made with 200 ml BBS-3 (Beckman; Southfield, Mich.), 84 ml Liquiflor (New England Nuclear; Boston, Mass.) and 2 l. toluene. No quenching by the insoluble cell debris was detected.

Identification of intracellular products. As reported previously,[32] the assay system primarily detected phosphorylated cytarabine compounds with human lymphocytes. However, some lymphocytes from the other species retained free cytarabine (and in

TABLE 1. SURVEY OF CYTARABINE INCORPORATION AND CONVERSION TO PHOSPHORYLATED METABOLITES BY SMALL LYMPHOCYTES ISOLATED FROM COMMON SPECIES*

Species	Thymus			Spleen			Lymph node			Blood		
	Incorporated		Phosphorylated	Incorporated		Phosphorylated	Incorporated		Phosphorylated	Incorporated		Phosphorylated
	Individual	Av.	Av.	Individual	Av.	Av.	Individual	Av.	Av.	Individual	Av.	Av.
Mouse	7·5, 8·0, 8·3, 9·6, 10·2, 10·7, 11·3, 11·5	9·6	7·8	2·9, 3·2, 3·3, 3·8, 4·2	3·4	1·3	1·3, 3·6, 3·8	2·9	1·2	4·0, 4·0, 4·8	4·2	3·0
Rat	7·7, 8·0, 8·3, 9·4, 9·5, 9·5, 10·5	8·9	7·4	2·3, 2·5, 2·5, 3·2, 4·1, 4·2, 4·3, 4·3	3·4	1·9	3·1, 3·1, 3·9, 4·7	3·7	1·8	4·0, 4·2, 4·2, 5·1	4·3	2·1
Dog	10·4, 14·5, 20·6	15·1	12·4	1·6, 1·7, 1·8, 1·8	1·7	0·7	1·4, 1·6, 1·6, 1·8	1·6	0·7	1·3, 1·7, 1·8, 1·8	1·6	0·6
Rabbit	19·6, 20·4, 22·1, 28·3, 29·1	23·9	20·2	2·8, 2·8, 3·0, 4·0	3·1	1·2	3·5, 3·8, 5·0, 6·2	4·6	3·6	4·8, 5·5, 5·9, 6·4	5·6	3·6
Rhesus monkey	13·7, 13·7, 14·7 17·1	14·8	11·4	3·9, 5·9, 7·9, 9·9	6·9	5·5	2·7, 4·7, 5·4, 7·0	4·9	4·4	4·0, 4·1, 4·1, 4·2, 5·2, 5·3	4·4	2·5
Man	11·0, 11·1, 14·8, 16·3	13·3	12·6	Not done			Not done			10·2, 10·2, 11·1, 12·5, 16·4	12·4	11·4

* The results are expressed as picomoles incorporated or phosphorylated per 10^6 lymphocytes per 15 min. The difference between the two average values for each organ represents free cytarabine, with the exception of rhesus monkey blood and spleen lymphocytes in which *ara*-uridine was also found.

one case, the deaminated metabolite, *ara*-uridine). For this reason, the radioactive intracellular components were identified by thin-layer chromatography (TLC). After the 5-min saline incubation described above, the cells were resuspended in 0·1 ml saline and the triplicate samples were pooled and frozen at −70°. Control experiments showed that the cells released all their intracellular radioactivity upon thawing. After centrifugation, the supernatants were collected, the pellets were washed, the washes were pooled together with the supernatant, the supernatants were concentrated by evaporation and applied to TLC plates (Brinkman Instruments). The reference standards included cytarabine, *ara*-uridine, *ara*-cytidine monophosphate (*ara*-CMP) and pyrimidine nucleoside triphosphates. The first TLC system consisted of methylethylketone–acetone–water in a ratio of 7:2:1. *Ara*-CMP remained at the origin, cytarabine moved only slightly, while *ara*-uridine migrated with an R_f value of 0·42. The second system consisted of isopropanol–NH_4OH–water in a ratio of 7:1:2. Cytarabine phosphates remained near the origin, while cytarabine and *ara*-uridine possessed R_f values of 0·55 and 0·60 respectively. Thus, the latter system allowed direct computation of the per cent of cytarabine phosphates, while the per cent of free cytarabine could be determined by subtracting the per cent of *ara*-uridine (determined in the first system) from the combined per cent of *ara*-uridine and cytarabine (determined in the second system).

The only lymphocytes which formed an appreciable amount of *ara*-uridine under the assay conditions described were those from rhesus monkey blood and spleen. Expressed as the per cent of total radioactivity after incubation, the preparations had, respectively, 41 and 18% *ara*-uridine. The other lymphocyte extracts had about 5 per cent or less of the total radioactivity in the region of *ara*-uridine migration, a value which approached the limits of detectability. More significantly, nonprimate lymphocytes contained appreciable amounts of free cytarabine after incubation. Furthermore, there appeared to be an organ differential: thymus lymphocytes from the nonprimates all had about 15 per cent free cytarabine after incubation, whereas the other organ lymphocytes had much higher values, ranging from 25 to 50 per cent free cytarabine. The lymphocytes from rhesus monkeys and man contained essentially no free cytarabine after incubation. Although it was not possible to perform TLC on every individual sample because of the limits of detectability and the limited supply of some lymphocytes, this was not crucial for the following reason. The main factor determining differences (or similarities) between lymphocytes was the amount of intracellular radioactivity incorporated during incubation. Therefore, Table 1 displays the incorporation data from individual experiments, the average incorporation and the average amounts of phosphorylated cytarabine. As noted above, with the exception of the rhesus monkey spleen and blood lymphocytes which contained *ara*-uridine, the amount of free cytarabine retained can be computed by simply subtracting the two averages.

DNA synthesis (measured by thymidine incorporation) of normal and phytohemagglutinin-stimulated cells. The procedure of Valentine[38] was used with the following slight modifications. Purified phytohemagglutinin (PHA, 24·8 mitogenic units/2 mg vial, Wellcome Reagents Ltd, Beckenham, England) was added, 8 μg total, to culture tubes containing 2×10^6 lymphocytes. The thymidine (thymidine-2-^{14}C, 54 mc/mmole, New England Nuclear Corp.; Boston, Mass.) was added, 0·1 μc/tube, either at the start of incubation or after 64 hr in culture. In both cases, the incorporation of thymi-

dine was stopped after 3 hr by the addition of ice-cold 10% trichloroacetic acid (TCA). After successive TCA and methanol washes[38] and after drying, the pellet was dissolved overnight at room temperature with 1·0 ml Hyamine (Packard Instrument Company; Downers Grove, Ill.). The dissolved material was then transferred to counting vials using 3–5 ml washes of Diotol (Burdick & Jackson Laboratories; Muskegon, Mich.). The results, Table 2, are expressed as counts per minute incorporated per culture tube (which contained 2×10^6 cells at the start of incubation).

TABLE 2. DNA SYNTHESIS (MEASURED BY THYMIDINE INCORPORATION) OF NORMAL AND PHA-STIMULATED CELLS

Cells*	Thymidine incorporated (Counts/min culture tube) From 0–3 hr	From 64–67 hr −PHA	From 64–67 hr +PHA	Cytarabine phosphorylated† (pmoles/10^6 cells/15 min)
Rat thymocytes	2600	10	20	7·4
Rabbit thymocytes	1100	10	50	20·1
Human blood lymphocytes	60	300	7500	12·6

* The cells were isolated by the Hypaque-Ficoll procedure described in Experimental.

† These data are from Table 1.

RESULTS AND DISCUSSION

The discussion and interpretation of these results, summarized in Table 3, requires cognizance of the following considerations. First, the lymphocytes were all "small", as opposed to medium and large lymphocytes, and varied in average diameter from 6·5 to 7·8 μ (a separate report will show that there was no correlation between size and phosphorylating activity), and they were all morphologically similar. Second, the lymphocytes studied in this report were operationally defined in that they consisted of that fraction of the total population which was isolated by the Hypaque-Ficoll procedure. Third, the data from the spleens and lymph nodes should be compared somewhat cautiously because these tissues are more heterogeneous with respect to lymphocyte type (compared with the thymus and blood), the lymph nodes are probably more reflective of local antigenic stimuli and the lymph nodes were obtained from different anatomic locations. Finally, it should be noted that the term "thymus-derived lymphocyte" means expressly those lymphocytes isolated from the thymus and does not imply that these cells are solely responsible for the so-called thymus-dependent immune responses. This qualifying statement is necessary because the relationship between the two is not exactly known at this time.

The first major finding is that the results were consistent with the hypothesis[27] that adamantoyl cytarabine primarily suppressed thymus-dependent immune responses in rats, i.e. the thymus-derived lymphocytes phosphorylated cytarabine approximately 4-fold more than those obtained from the other organs of the rats. As an aside, if

TABLE 3. COMPARISON OF THE ABILITY OF LYMPHOCYTES TO PHOSPHORYLATE CYTARABINE RELATIVE TO HUMAN BLOOD LYMPHOCYTES

Species	% Human blood lymphocytes*			
	Thymus	Spleen	Lymph node	Blood
Mouse	68	11	11	26
Rat	65	17	16	18
Dog	109	6	6	5
Rabbit	176	11	32	32
Rhesus monkey	100	48	39	22
Man	111	Not done	Not done	100

* Calculated from the phosphorylation data in Table 1.

cytarabine does exert its major effects on thymus-dependent responses, it might be expected that synergistic immunosuppressive effects would be observed in combination with an immunosuppressant which has a different mechanism of action and, indeed, such results have been claimed with cytarabine and anti-spleen lymphocyte globulin in rats.[39]

The second major finding is revealed by comparing the results between the organs within one species. With one notable exception, the lymphocytes obtained from the thymus were considerably more active than those obtained from any other organ. The smallest differences between the thymus and blood lymphocytes were observed in the rodents and the rhesus monkeys (the thymus lymphocytes were from 2·5 to 5·5 times more active) and the largest difference was observed in the dog (about 20-fold). The notable exception was man in whom no difference between thymus and blood lymphocytes can be detected. It should be recognized, however, that the values for the human thymus lymphocytes probably represent minimum values, since these tissues were not collected and tested for activity on the same day due to the time required for shipping.

Although the thymocyte–blood lymphocyte differential could be superficially explained on the basis of a correlation with DNA synthetic capacity (the more DNA synthesis, the more phosphorylation) because the thymus is composed of primarily rapidly dividing cells,[40] several factors argue against such a simple interpretation. First, as the results in Table 2 show, there was absolutely no correlation between the inherent DNA synthetic capacity of a cell type, or that observed after PHA stimulation, and the ability of that cell to phosphorylate cytarabine. Second, Durham and Ives[18] did not obtain an exact correlation of deoxycytidine kinase activity and cell proliferation rate. Furthermore, only cells passing through the S-phase of the cell cycle appear to be sensitive to cytarabine[41–46] and clearly the human blood lymphocytes were not passing through this phase as evidenced by the extremely low amount of thymidine incorporated.

The third major finding is revealed by comparing the results between the species. With the exception of mice and rats (in which the results were essentially identical), the lymphocytes from each species possessed phosphorylating capabilities unique to that species.

The results in mice confirm what might have been predicted from the studies of Saslaw *et al.*,[47] who commented that mouse thymus retained radiolabeled cytarabine for longer periods of time compared with other organs, including the spleen and from those of Lenaz *et al.*,[44] who found that cytarabine suppressed DNA synthesis in mouse thymus more than in spleen. Similarly, Durham and Ives,[18] in studying the tissue distribution of deoxycytidine kinase in both mice and rats, showed that the thymus contained the highest activity compared with other organs.

The dog spleen, lymph node and blood lymphocyte had the lowest activity of any lymphocytes of any species (although the thymus had "normal" activity). If the basic tenet of these studies is correct, i.e. the phosphorylating ability of a lymphocyte determines its sensitivity to cytarabine, then it is tempting to speculate on the types of immunosuppressive specificity that might be obtained after cytarabine administration in the various species. Thus, the dog might provide the most sensitive model for studies on immunosuppressive specificity because the greatest differential (20-fold) between the thymus-derived lymphocytes and the other lymphocytes was observed in this species.

The most active lymphocytes of any species examined to date were those isolated from rabbit thymus (which means it would probably be an excellent tissue source for purification of deoxycytidine kinase and similar enzymes). The rabbit thymocytes were about twice as active as those of man, monkey and dog and to some surprise, three times as active compared with those of the other two rodent species.

The primary distinguishing feature of the rhesus monkey was that this was the only species whose lymphocytes formed appreciable amounts of *ara*-uridine. It should be noted, however, that this was observed with the blood and spleen lymphocytes only and not with the thymocytes or lymph node lymphocytes.

The main distinguishing feature of man is obvious—the blood lymphocytes were the most active of any species, including the rhesus monkey. Human blood lymphocytes were from 4 times (compared with rhesus monkey blood lymphocytes) to 20 times (compared with dog blood lymphocytes) more active than those of the other species.

These data bear on a highly theoretical and speculative point, viz. what is the significance of human lymphocyte phosphorylation if the lymphocyte is not actively engaged in synthesizing DNA and if it is not actively proliferating. One obvious possibility is that the lymphocyte simply carries its own self-destruction mechanism until that time when it becomes activated (e.g. by the appropriate antigen or during leukemogenesis). Interestingly, there is some indirect evidence to support such a speculative suggestion. First, specific lymphopenia has never been specifically associated with cytarabine therapy in man, whereas a general suppression of other bone marrow-derived elements has been noted.[1–5] Second (with the proviso that the leukemic cell can phosphorylate cytarabine, as already noted), leukemic cells can be suppressed by cytarabine so that the sensitivity of these cells compared to normal lymphocytes could be explained in that the former are actively proliferating whereas the latter are not. Third, and perhaps the most tenuous point, is that this speculation fits with the observations of Mitchell *et al.*[12] That is, in man, immune responses which are probably primarily dependent on lymphocyte proliferation (primary and secondary antibody responses and the primary delayed hypersensitivity response) were suppressed by cytarabine, whereas immune responses which probably do not require extensive lymphocyte proliferation

(established secondary delayed hypersensitivity reactions) were not suppressed by cytarabine.

Finally, considerable effort has been expended in performing immunosuppressive experiments in animals with cytarabine[20,21,35,36,39,48–56] and various derivatives.[22,23,25–27] If the major determinant of cytarabine immunosuppressive specificity is, indeed, phosphorylation by small lymphocytes, then the relevance of previous animal immunosuppressive studies, including our own, would seem to be somewhat in jeopardy, since the current results suggest that the immunosuppressive specificities observed in animals during cytarabine (or cytarabine derivatives) therapy might be different from those obtained in man.

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