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Whole Blood Lymphocyte Microculture Carried Out By A Simple Kit*

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During the last years several micro-methods have been proposed to investigate the blastic transformation of peripheral-blood lymphocytes after mitogen or MLC stimulation.[1-13]

It obvious that economy in the blood amount employed to carry out blood tests is important owing to the fact that the number of tests requesed to the laboratory is progressively increasing. Furthermore, micro methods are often a need in childhood, as well as for investigation on small experimental animals, mostly when the single animal can't be sacrificied but has to be tested before, during and after given treatments.

In this paper we will discuss the results of our further study on the blastic transformation of peripheral-blood lymphocytes from either human subjects or different experimental animals, by testing the pheno-

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menon in the short-term cell culture set up in a fully preprepared kit. This allows to perform a quite simple micro-test, in which only few drops of whole blood may be used.

Material and Methods

The Kit: The kit used in these experiments (Blasto-Kit I.S.M.) consists of vials containing a lyophilized mixture made up by RPMI 1640 Hepes buffered, suplemented with appropriated antibiotics; AB-pool serum (AB-pool Cytoculture I.S.M.); PHA at the optimal dose, selected via dose-response curve. The kit has also blank control vials, where the lyophilized mixture is not supplemented with any mitogen. Finally, the kit is provided with ampules of proper solvent, to make the mixture solution when the culture has to be set up.

The blood lymphocytes tested: The extent of peripheral blood lymphocyte transformation of 20 healthy human donors was studied in morphology. Again, the extent of ^{3}H-Tdr incorporation (c.p.m.) of 30 further healthy donors was investigated. All them were volunteers ageing between 20 and 50 years, both sexes (students or Lab-workers, including ourselves).

Also lymphocyte transformation of experimental animals were studied both in morphology and via the ^{3}H-Tdr incorporation. In particular, the following animals were used:

Ten rabbits, domestic chinchila strain: both sexes, 1-3 years old.

Nine guinea pigs, usual laboratory strain of the Dept. of Biochemistry of the Medical Faculty of Novi Sad: both sexes, 5-6 months old, weight about 750 g.

Six hamsters, strain AURA from the Central Institut für Versuchistierzuch, Hannover: both sexes, 4-6 weeks old, weight 60-80 g.

Nine rats, Wistar strain: both sexes, 3 months old, weight 160-210 g.

Nine white laboratory mice, strain of the Dept. of Biochemistry of the Medical Faculty of Novi Sad: both sexes, 8-10 weeks old, weight 20-30 g.

Human peripheral-blood was collecled by a cubital venous puncture. Mouse blood was obtained from the retro-orbital venous sinus.

Rats, hamsters and guinea-pigs were anestetized with ether before bleeding, and their blood was obtained via heart puncture, and collected into heparinized disposable plastic ysringes. Rabbit blood was drawn from the marginal ear vein.

Lymphocyte Cultures: Our study has been carried out both in morphology and via evaluation of ^{3}H-Tdr incorporation. To set up the cultures, first the mixture in both the mitogen and the blank control vials was diluted with 2.5 ml of the proper solvent. Then, 5 drops of whole peripheral-blood (200 µl) were added to each vial for evaluation in morphology; and drops (120 µl) for the study on the ^{3}H-Tdr incorporation. These blood quantities were chosen on a dos-esponse curves preliminary sought by testing different blood quantities, from 2 to 10 drops (80-400 µl).[9, 12] Finally, the culture vials were incubated in an incubator at 37°C.

After 3-days incubation at 37°C the kit culture vials were harvested. For the morphological study for this, at the end of the incubation period the cells are spun down by centrifugation at 400 g for 10 min, and the cell pellet was smeared. The smears were fixed and stained with May Grünwald-Giemsa or Romanovski dye for differential counts. Two hundred lymphatic cells (non transformed lymphocytes and blast-like cells) were examined on each smear, and the number of macrophages observed (during) per one hundered lympocyte count was also recorded. Finally, the blastic index was calculated: this being the ratio between the blast percentual extent observed in the Blasto-kit PHA culture, and the extent observed in the Blasto-kit control culture.

The extent of the ^{3}H-Tdr incorporation by human lymphocytes was evaluated with a liquid phase scintillation counter. After 52-54 hours of incubation at 37°C, the kit-culture vials were suplemented with ^{3}H-Tdr (spec. act. 200 mCi/mml), at a concentration of 1 µC/ml. Then, 18-20 hours later (72 hours after the culture onset), the cells were collected on Whatman GF/C glass fiber filter paper by means of a vacuum pump. The filters were washed with 5 ml distilled water, 5 ml absolute methanol. The filters were placed in counting vials, and dried 30 min at 50°C. Subsequently, 5 ml of liquid scintillation solution (consisting of toluene with PPO 5 g/l, and dimethyl-POPOP 100 mg/l) were added and each vial was placed in a liquid phase scintillation counter for 10 minutes. The mean values of replicate cultures under the same experimental conditions are expressed in c.p.m.

For the observation on the the lymphocytes from experimental animals, the kit-culture vials were supplemented with ^{3}H-Tdr (spec. act. 5 Ci/mM) at a concentration of 1 µC/ml just 48 hours after the incubation onset. Then, 24 hours later (72 hours after the culture onset), incubation was interrupted by putting the tubes in an ice bath. The cultures were washed witn cold saline, then precipitated with 5 % trichloroacetic acid (TCA), and the precipitates washed three times with

5 % TCA. The precipitates were then solubilized at 55°C using Beckman tissue solubilizer (BTS), and blanched with 30 % H_2O_2. Then, 5 ml of liquid scintillation solution (consisting of toluene with PPO 5 g/l and POPOP 0.2 g/l) and 0.05 ml of the solubilized precipitates were added to each vial, and the samples were counted in a liquid scintillation spectrometer Beckman LS-250 for 10 min. Here also, the mean values of replicate cultures under the same experimental conditions are expressed in c.p.m.

Statistical Evaluation of the Results: The following results have been statistically evaluated: (a) the extent of ^{3}H-Tdr incorporation in the PHA-Blasto-Kit cultures in relationship to the extent of ^{3}H-Tdr incorporation in the control Blasto-Kit-cultures; (b) the percentage frequencies of the transformed lymphocytes (blast-like cells) in the control cultures in relationship to the corresponding data in the PHA-Blasto-Kit cultures; (c) the blastic indices (ratio between the values in the PHA-Blasto-Kit cultures and those values in the control cultures). Means, standard deviations (S.D.), standard errors (S.E.), the percentage coefficient of variability (C.V. %) for each parameter have been calculated.

The results obtained in the control cultures and those observed in the PHA-ones have been compared by both t-test and F-test.

Results

The average of the lymphocyte transformation extent expressed either in c.p.m. or in blast percentages observed for the different tested beings, are quoted in Table I. A marked difference between the extent of lympocyte transformation in the PHA Blasto Kit cultures and that extent in the control ones is evident both in the scintillation counts and in morphology, for all the tested donors. These differences were significant for both to the t-test and to the F-test. Thus, discrimination indices point out a clear cut effect of the mitogen in the experimental model we have tested.

On the other hand, our results show rather high spontaneouslymphocyte transformation also in the control cultures of the tested experimental knimals, which mostly appears from the observations in morphology, whereas spontaneous transformation in the control cultures of human lymphocytes is much lower. This might be related to possible intrinsic feature of the lymphocytes of laboratory animals.

Observations in morphology have also provided information on the cell appearance. The microscope examination of the culture smears

TABLE I

EXTENT OF THE LYMPHOCYTE TRANSFORMATION IN THE DIFFERENT TESTED DONORS
(n.p. = non performed; C. V. % = percentual coefficient of variability)

Species of the Lymphocyte Donors	Para-meters	Blastokit Control		Blastokit PHA		Statistical Significance between control and PHA data		
		Morphol. (Blasts %)	Scintill. (Blasts %)	Morphol. (Blasts %)	Scintill. (c. p. m.)	Test	Morphol.	Scinitll.
Human	Average	2.32	486.37	80.20	83049.09	t	+++	+++
	S.D	2.83	155.25	8.69	50755.23	F	+++	+++
	S.E.	0.63	28.42	1.94	9266.60			
	C.V.%	121.75	31.92	10.84	61.11	D.F.	1.38	1.58
Rabbit	Average	22.93	1563.00	78.93	76157.61	t	+++	+
	S.D.	4.88	806.25	7.10	37600.23	F	+++	+
	S.E.	1.84	465.49	2.68	21708.50			
	C.V.%	21.27	51.58	9.00	49.37	D.F.	1.12	1.40
Guinea Pig	Average	32.00	1593.60	84.42	48975.89	t	+++	++
	S.D.	5.11	699.46	1.50	10969.33	F	+++	++
	S.E.	2.09	403.83	0.61	6333.14	D.F.	1.10	1.40
	C.V.%	15.97	43.89	1.77	2.40			
Hamster	Average	33.80	n.p.	74.50	n.p.	t	+++	
	S.D.	5.11		5.53		F	+++	
	S.E.	2.09		2.26		D.F.	1.10	
	C.V.%	15.45		7.43				
Rat	Average	37.52	n.p.	66.75	n.p.	t	+++	
	S.D.	3.39		3.34		F	+++	
	S.E.	1.38		1.36				
	C.V.%	9.09		5.01		D.F.	1.10	
Mice	Average	33.42	n.p.	74.67	n.p.	t	+++	
	S.D.	4.53		4.24		F	+++	
	S.E.	1.85		1.73				
	C.V.%	13.56		5.68		D.F.	1.10	

n.p. = non performed; - not significant; + significant 5 %; ++ significant 1 %; +++ significant 0,1 %

in general did show rather good preparations. They showed quite numerous erythrocytes, mostly grouped or conglutinated together. Lymphocytes either non-transformed or transformed were randomly distributed in the microscopic field. On the other hand also dense groups of lymphocytes and more or less numerous macrophoges were observed.

Morphology of the transformed lymphocytes did not show marked differences among the different test animals. Nevertheless, mostly the rabbit and guinea pig transformed lymphocytes were similar to those from human peripheral blood. Indeed, those transformed lymphocytes appeared as large blast-like cells, with enlarged nuclei frequently provided with one or more numerous well engraved nucleoli. Those nuclei were surrounded by large basophilic-pyroninophilic cytoplasm, sometimes showing more or less numerous vacuoles. Finally, several of the above-mentioned blast-like cells were in mitosis and this not only in the human lymphocyte cultures, but also in the experimental animal cultures.

Concerning macrophages, they appeared particularly frequent in the rat and hamster cultures, either in the control ones (means 12.00 and 10.45 %, respectively), or in the PHA ones (means 10.18 and 28.09 %, respectively). Granulocytes were rather numerous in mouse cultures.

Disiscuson

The results of this study show that peripheral-blood lymphocytes from mouse, rat, hamster, guinea-pig, and rabbit may undergo intense blastic transformation in the short-term cell culture like human peripheral-blood lymphocytes, when properly cultivated and submitted to mitogen stimulation. It appears that this may well occur also by using appropriate whole-blood culture microtechinique, and that Blasto-Kit is quite helpful for this purpose. Indeed, the micromethod which may be performed with Blasto-Kit offers an adequate discrimination index to evaluate the lympocyte transformation extent between the PHA stimulated cultures and the non-stimulated controls (blastic index), despite of a rather high spontaneous lympocyte transformation which may occur in the Blasto-Kit control cultures of the examined exeprimental animals.

The simplicity of the method makes the investigation on the transformation capability of lymphocytes easy to carry out in every laboratory. Again, the small quantity of blood which is needed to perform the test offers important advantages: owing to the progressively

increasing number of tests requested to the laboratory the cut down in the amount of blood employed for each single test becomes a need for adult patients, and often a "must" in childhood, as well as in small experimental animals mostly when each single subject has to be tested before, during, and after given treatments.

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