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TIME FACTOR IN PHA RESPONSIVENESS OF HUMAN BLOOD LYMPHOCYTES AFTER IN VITRO IRRADIATION

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Previous investigations (BARAL & BLOMGREN 1976, SCHREK & STEFANI 1964, CIRKOVIC 1969, BRAEMAN & MOORE 1974) have demonstrated that exposure of human peripheral lymphocytes to increasing doses of roentgen rays reduces their PHA-responsiveness as a two-phased function. A steep fall of the PHA-reactivity is recorded up to 8 Gy, followed by a plateau phase at higher doses. This observation may be interpreted in several ways. One possibility could be that there exists a true difference in sensitivity to radiation between two lymphocyte subpopulations. An alternative explanation could be that there are two subpopulations of PHA-reactive cells: one which is activated rapidly by PHA and thereby builds up a repair system for the structures injured by irradiation, and another subpopulation which is activated more slowly by PHA and that dies in interphase.

In this investigation this question has been explored by varying the sequence and time intervals between radiation exposure and PHA-stimulation.

Material and Methods

Blood samples were obtained from healthy volunteers. Venous blood was drawn in heparinized syringes, and nucleated cells were separated be centrifugation on Ficoll-Isopaque (BÖYUM 1968). The cells were washed twice by centrifugation in Eagle's Minimal Essential Medium supplemented with Earle's salts (MEM). Of the cells, 90 to 95 per cent had the morphology of small lymphocytes, the remainder being

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classified as monocytic or granulocytic cells. Cell preparations (1.0×10^6 cells/ml) were suspended in MEM supplemented with penicillin, streptomycin and 10 per cent of heat inactivated human serum (HS). Cultures that received PHA were incubated with this agent for 6, 12, 24, 48, and 72 hours in a humidified 5 per cent CO_2-air atmosphere. The cells were then washed by centrifugation and transferred to glass Petri dishes and irradiated. After irradiation, the cells were washed once in MEM and placed in the wells of plastic microtest plates containing 0.2 ml of medium and 1×10^5 cells. All cultures were set up in quadruplicate. PHA was added to some of the cultures and others served as controls. After four days of incubation at 37°C in a humidified 5 per cent CO_2-air atmosphere, each culture received 1 μCi ^{3}H-thymidine (5 Ci/mM, Radiochemical Center, Amersham, England). Twenty-four hours later incorporated activity was determined (LILLIEHÖÖK & BLOMGREN 1974) and expressed as counts per minute (cpm). Other lymphocyte preparations from each donor were irradiated before PHA stimulation. The cells were washed once after irradiation and thereafter incubated in MEM with HS and antibiotics for 1, 12, 24, 48, and 72 hours. The cells were then cultured with PHA in microtest plates as described. Trypan-blue exclusion was used to determine cell viability. Isotope uptakes of unstimulated cultures were subtracted from those obtained in corresponding PHA stimulated cultures taken from the same donor. Mean values of quadruplicate cultures were calculated on an arithmetic basis. Stimulation of irradiated cultures was expressed as the percentage of incorporation of ^{3}H-thymidine in comparison with unirradiated controls.

Irradiation of cell suspensions was performed at room temperature as described previously (BARAL & BLOMGREN 1976) with Siemens equipment operated at 140 kV and 20 mA. The doses employed were 2, 4, 8, 16, and 32 Gy.

Lymphocytes were stimulated with phytohaemagglutinin (PHA, Bacto-phyto-haemagglutinin M, Difco Lab. Detroit, Mich. USA). The contents of commercially available vials were dissolved in 5 ml of MEM (100 per cent of PHA). This solution was further diluted to a final concentration of 3 per cent, which has previously been shown to yield optimum DNA-synthetic responses of human lymphocytes (BLOMGREN 1974).

Results

The results of one representative experiment (Fig. 1) demonstrate that the dose response curves are essentially similar for lymphocytes which were stimulated with PHA for 1 to 24 hours following irradiation in vitro. A profound decrease of reactivity was found in a large fraction of cells in the dose interval 1 to 8 Gy, while a smaller fraction was only slightly affected by increasing the dose from 8 to 32 Gy. When the time between irradiation and the subsequent stimulation was extended to 48 to 72 hours no 'resistant' cell fraction was detected. The stimulations, expressed as cpm, of the non-irradiated PHA-stimulated cultures are presented in Table 1.

The proportion of the resistant cells decreased somewhat when the lymphocytes

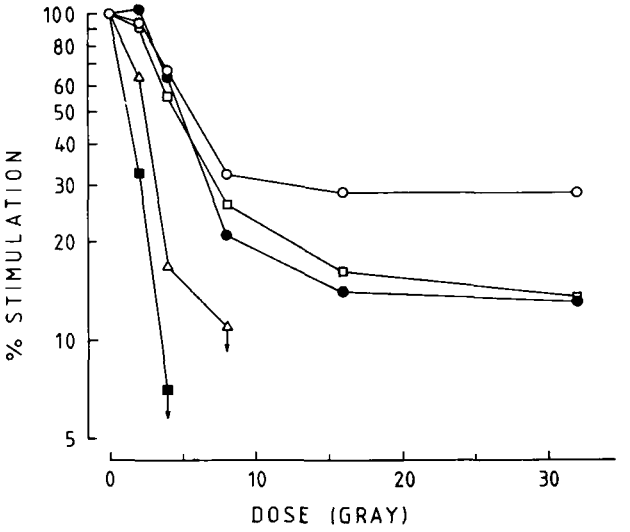

Fig. 1. Relative ^{3}H-thymidine uptakes of irradiated lymphocyte preparations exposed to 3 per cent of PHA at different times following irradiation. PHA added ○ 1 hour, ● 12, □ 24, ■ 48 and △ 72 hours after irradiation. Arrows indicate that stimulation of the cells was less than 0.5 per cent.

were cultured with PHA for different times between 12 and 72 hours before irradiation (Fig. 2). For comparison, an experiment in which the cells were PHA-stimulated 1 hour after irradiation is also illustrated. The stimulation of the unirradiated cultures as well as the calculated radiation resistant cell fractions are listed in Table 2. This cell fraction was estimated by the point of intersection between the linear regression line ($y = kx + L$) of the observations making up the plateau of the curves and the ordinate. Two observations emerge from this table: the absolute stimulations were decreased by prior exposure of the cells to PHA and the fraction of radiation resistant cells decreased by preincubation with PHA.

Discussion

The majority of cells which are activated to DNA-synthesis and subsequent mitosis by PHA are T-cells (GREAVES & JANOSSY 1972). This subset of the lympho-

Table 1

PHA-stimulations of non-irradiated cell cultures presented in Fig. 1

Time between irradiation and PHA-stimulation (hours)	^{3}H-thymidine incorporations (mean cpm)
1	255 000
12	272 000
24	281 000
48	348 000
72	186 000

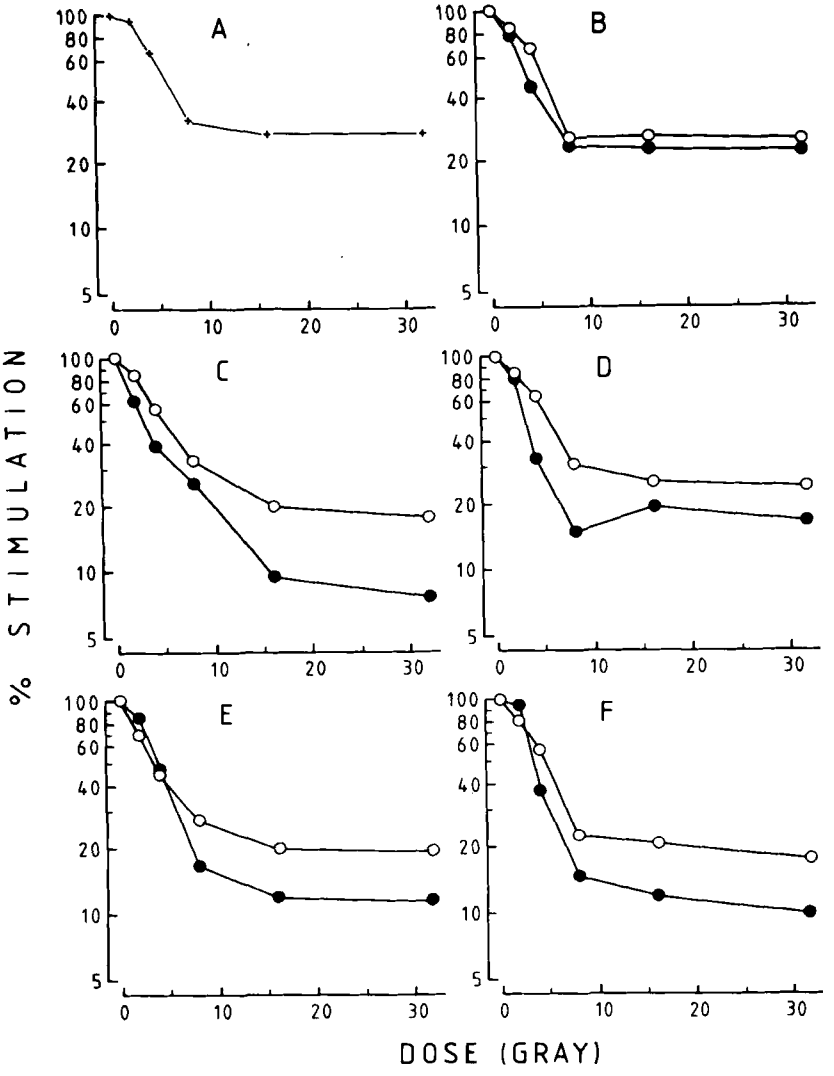

Fig. 2. Relative ^{3}H-thymidine uptakes of irradiated lymphocyte preparations cultured with 3 per cent PHA for different times before irradiation. A—PHA added 1 hour after irradiation. B—6, C—12, D—24, E—48 and F—72 hours incubation with PHA before irradiation. ● PHA pretreatment. ○ Control cultures incubated for the corresponding times in MEM and stimulated with PHA immediately after irradiation.

cyte population may be divided into two groups differing in sensitivity to ionizing radiation in vitro (SCHREK & STEFANI, CIRKOVIC, BRAEMAN & MOORE, BARAL & BLOMGREN). However, in a previous report (BARAL et coll. 1977) the proportion of radiation resistant PHA-reactive cells was not increased in the blood of patients with malignancy after radiation therapy in spite of the fact that the lymphocyte

Table 2

PHA-stimulations of non-irradiated cell cultures and proportions of 'resistant' cells of the lymphocyte preparations presented in Fig. 2

Time between PHA-stimulation and irradiation (hours)	Pretreatment	^{3}H-thymidine incorporations (mean cpm)	Fraction of resistant cells[1] (per cent)	Radiation doses for estimating the resistant cell fraction (Gy)
6	stimulated	101 000	24	8–32
	unstimulated	121 000	26	8–32
12	stimulated	54 000	11	16–32
	unstimulated	278 000	35	8–32
24	stimulated	146 000	16	8–32
	unstimulated	261 000	32	8–32
48	stimulated	53 000	17	8–32
	unstimulated	200 000	28	8–32
72	stimulated	44 000	16	8–32
	unstimulated	191 000	24	8–32

[1] The resistant cell fraction was estimated by calculating the linear regression line ($y = kx + L$) of the stimulations obtained in cultures exposed to the indicated doses of radiation. The value obtained by extrapolation to 0 Gy has been taken as the fraction of resistant cells.

number was reduced to less than 50 per cent. Thus, there was not a selective elimination of 'sensitive' lymphocytes as could be expected. This finding argues against the existence of two distinct groups of PHA-responsive lymphocytes differing in sensitivity to radiation.

One possible explanation for the two-phased dose response curve of PHA-responsive peripheral lymphocytes could be that this subpopulation contains one group of cells which are activated rapidly and another more slowly by PHA. Cells of the first group could theoretically survive the radiation injury due to the rapid build-up of repair enzymes during PHA-activation. This thesis is supported by the finding that PHA-activated lymphocytes exhibit significantly elevated DNA-polymerase and ligase activities (LOEB et coll. 1968, LOEB & AGARWAL 1971, AGARWAL & LOEB 1972, PEDRINI et coll. 1972). Moreover, HASHIMOTO et coll. (1975) have observed a ten-fold increase of rejoining of single strand DNA-breaks in PHA-activated lymphocytes compared with non-transformed. The report by SCHREK & STEFANI demonstrating an increased resistance of PHA-activated lymphocytes supports this view.

This investigation, employing human peripheral lymphocytes, has failed to demonstrate any increase of the radiation resistant fraction of PHA-responsive lymphocytes after exposure of the cells to the lectin for time periods ranging from 6 to 72 hours. If anything, the radiation resistant fraction decreased by prior

cultivation of the lymphocytes with PHA. The present data are in agreement with those showing that the PHA-response of lymphocyte preparations decreases as the time interval between radiation and exposure to PHA is increased (SCHREK & STEFANI). However, a surprisingly large proportion of cells were able to respond to PHA 24 hours after irradiation.

Various proliferating mammalian cells have been shown to become progressively more sensitive to radiation as they proceed towards mitosis (TERASIMA & TOLMACH 1963, SINCLAIR & MORTON 1966, SINCLAIR 1968). Theoretically, this could explain the biphasic dose-response curve, since lymphocytes in the blood may be in different phases of the cell cycle. This possibility seems unlikely considering the fact that the radiation sensitive fraction is at least three times greater than the resistant fraction and that less than 0.5 per cent of peripheral blood lymphocytes are in the S-phase. However, it is logical to expect that a larger proportion of lymphocytes approaches the radiation sensitive phases of the cell cycle following PHA-activation. This was found to be the case in the present series.

In conclusion, the results of this investigation do not give any support for the view that PHA-stimulation of lymphocytes can rescue them from a death induced by radiation.

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SUMMARY

Human blood lymphocytes were irradiated in vitro prior to or following PHA-stimulation. Radiation dose-response profiles indicate that PHA-pretreatment of cells has no protective effect. PHA-reactivity of the cells decreases with increase of the time interval between irradiation and subsequent PHA stimulation.

ZUSAMMENFASSUNG

Menschliche Blutlymphozyten wurden in vitro vor oder im Anschluss an eine PHA-Stimulation bestrahlt. Die Dosis-Responskurven deuten darauf hin, dass PHA-Vorbehandlung der Zellen keinen Schutzeffekt hat. Die PHA-Reaktivität der Zellen fällt mit steigendem Zeitintervall zwischen Bestrahlung und nachfolgender PHA-Stimulation.

RÉSUMÉ

Des lymphocytes sanguins humains ont été irradiés in vitro avant ou après stimulation par PHA. Les profils de réponse en fonction des doses de radiation montrent que le prétraitement par PHA des cellules n'a pas d'effet protecteur. La réactivité des cellules PHA diminue quand l'intervalle de temps entre l'irradiation et la stimulation ultérieure par PHA augmente.

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