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RESPONSE OF HUMAN LYMPHOCYTES TO MITOGENIC STIMULI AFTER IRRADIATION IN VITRO

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External radiation therapy of patients with malignancies may induce a state of lymphopenia in the peripheral blood (GOSWITZ et coll. 1963, MILLARD 1965, MEYER 1970, ILBERY et coll. 1971, THOMAS et coll. 1971, STJÄRNSWÄRD et coll. 1971, MCCREDIE et coll. 1972, GLAS & WASSERMAN 1974, BLOMGREN et coll. 1974 b, c). It has not been definitely settled whether this cell reduction is due to the fact that lymphocytes are killed off by the radiation or whether they escape from the blood into the lymphoid organs or other parts of the body. However, the first explanation seems most likely, since irradiation of parts of the body which do not contain large blood vessels causes little or no detectable lymphopenia (CHEE et coll. 1974).

The blood lymphocyte population remaining after completion of radiation therapy for carcinoma of the breast, urinary bladder or prostate exhibits a highly reduced mitotic response to purified protein derivate of tuberculin (PPD) in vitro (GLAS & WASSERMAN, BLOMGREN et coll. 1974 b). One explanation for this fact is that lymphocytes which are responsive to this antigen are killed or inactivated by the irradiation. It has also been reported that radiation therapy reduces the relative in vitro responses of blood lymphocytes to polyclonal phytomitogens (GOSWITZ et coll., MILLARD, MEYER, THOMAS et coll., ILBERY et coll.). However, this statement has not been confirmed by others (MCCREDIE et coll., BLOMGREN et coll. 1974 b, c).

Conflicting results have also been published regarding the type of lymphocyte which is reduced after radiation therapy. Some authors have claimed that the fre-

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quency of T-lymphocytes decreases to a higher extent than non-T-lymphocytes—mainly B-lymphocytes—(STJÄRNSWÄRD et coll.) whereas others have obtained the reverse results (BLOMGREN et coll. 1974 b, c). (For a review of T-and B-lymphocytes, see GREAVES et coll. 1974.)

In the present report the responses of human peripheral lymphocytes to various specific and unspecific mitogens following irradiation in vitro are analysed.

Materials and Methods

Eight members of the staff, 20 to 60 years old, served as lymphocyte donors. Venous blood was drawn in heparinized syringes and the nucleated cells separated by Ficoll-Isopaque gradient centrifugation as previously described (JONDAL et coll. 1972). The cells were then washed twice by centrifugation in Eagle's Minimal Essential Medium supplemented with Earle's salts (MEM). Such cell suspensions, regularly containing 90 to 95 per cent of lymphoid cells, will hereafter be termed non-purified. In some experiments such preparations were depleted of phagocytic cells. This was performed by adding carbonyl iron powder to the suspensions. These particles, and hence also cells which ingested iron, were then removed by a magnet by the method of BLOMGREN (1974). The resulting suspensions, will hereafter be designated Fe-purified.

These preparations of highly purified lymphocytes were used for the preparation of cell suspensions enriched for T- and B-cells. Separation of T- and B-cells was performed using the method described by JONDAL (1974). In short, lymphocytes were incubated with sheep erythrocytes (SRBC). During this step SRBC will adhere to T-cells in an immunologically nonspecific way (BRAIN et coll. 1970, BRAIN & GORDON 1971). The cell suspensions were then suspended in fetal calf serum and centrifuged on a Ficoll-Isopaque gradient. Due to the higher density, the T-cell-SRBC-complexes will sediment whereas non-SRBC-adhering cells, mainly B-cells, will stay at the fluid interface. For simplicity the precipitating cells will hereafter be termed T-cells and the cells at the fluid interface B-cells. The T-cells were exposed to a solution of ammonium chloride to lyse SRBC before used for experiments. Using the capacity of T-cells to adhere SRBC as a marker (BLOMGREN 1974), it was found that non-purified and Fe-purified cell preparations contained 60 to 65 per cent of T-cells and the T- and B-cell preparations 90 to 95 and 10 to 15 per cent, respectively. The number of cells was determined in a Bürker chamber after crystal violet staining.

Various cell preparations, suspended in MEM at a concentration of 1.0×10^6 cells/ml, were poured into glass Petri dishes (6 cm × 6 cm) forming a fluid depth of 2 to 3 mm. They were then exposed to various doses using a Siemens machine. The physical factors were: 140 kV, 20 mA, 4 Al-added filter, HVL 0.45 mm Cu, focus-target distance 40 cm. The irradiations were performed at room temperature. The exposure rate, in the centre of the 16 cm × 16 cm beam used for simultaneous

irradiation of 4 Petri dishes, was determined by ionisation chamber measurements. The homogeneity of the beam was checked by photographic dosimetry. The average exposure rate in the suspension was determined to 113 R/min $\pm$ 10 per cent.

The well known inhomogeneity in the absorbed dose at the interface between the glass and the suspensions has been neglected since it concerns only a very thin layer (<50 μm) and the results are based on intercomparisons of suspensions irradiated under equal conditions.

Unless otherwise stated, each well contained 1.0×10^5 cells suspended in 0.2 ml of MEM supplemented with 10 per cent of heat inactivated human serum, penicillin and streptomycin. Half of the cultures received a stimulant and the others served as controls. The stimulants were: (1) phytohaemagglutinin (PHA, Bacto-Phytohaemagglutinin M, Difco lab., Detroit, Mich., USA), (2) poke weed mitogen (PWM, Grand Island Biological Co., N.Y., USA). The contents of commercially available vials of PHA and PWM were dissolved in 5.0 ml of MEM. These solutions will hereafter be termed 100 % of PHA and PWM, respectively. (3) Concanavalin A (ConA, Sigma Chemical Co., St. Louis, Mo., USA) was dissolved in MEM. The final concentration of ConA in the cultures is expressed as μg) ml. (4) Purified protein derivate of tuberculin (PPD tuberculin, RT 22, Statens Seruminstitut, Copenhagen, Denmark). (5) Allogeneic or syngeneic non-purified lymphocytes previously treated with mitomycin C as described previously (BLOMGREN et coll. 1974 a). Cultures receiving allogeneic cells, mixed lymphocyte cultures (MLC), contained 2.0×10^5 responding cells and the same number of stimulating cells.

After 4 days of incubation at 37°C in a humidified 5 % CO_2-air atmosphere, each culture received 1 μCi of ^{3}H-thymidine (The radiochemical Center, Amersham, England. Specific activity 5 mCi/mM). Twentyfour hours later the cultures were terminated by being placed at -20°C. When convenient, the cultures were thawed and the fluid of each well passed through a microfilter which retains particulate material. This procedure was performed with the aid of a semiautomatic multiple-sample processor (Skatron, Box 3401, Lierbyen, Norway).

The filters were then placed in vials containing scintillation fluid and the activity measured by a liquid scintillation counter. The culture conditions and measurements of activity have been described in detail by LILLIEHÖÖK & BLOMGREN (1974).

The activity was expressed as counts per minutes (cpm). Isotope uptakes of unstimulated control cultures were subtracted from those obtained in corresponding stimulated cultures. The activity of cultures containing irradiated cells was related to the values obtained in corresponding non-irradiated cultures. The mean values of triplicate cultures were calculated on an arithmetic basis and expressed as per cent stimulation.

Results

Responsiveness of irradiated lymphocytes to phytomitogens. Preparations of non-purified lymphocytes were exposed to various doses of irradiation. They were then

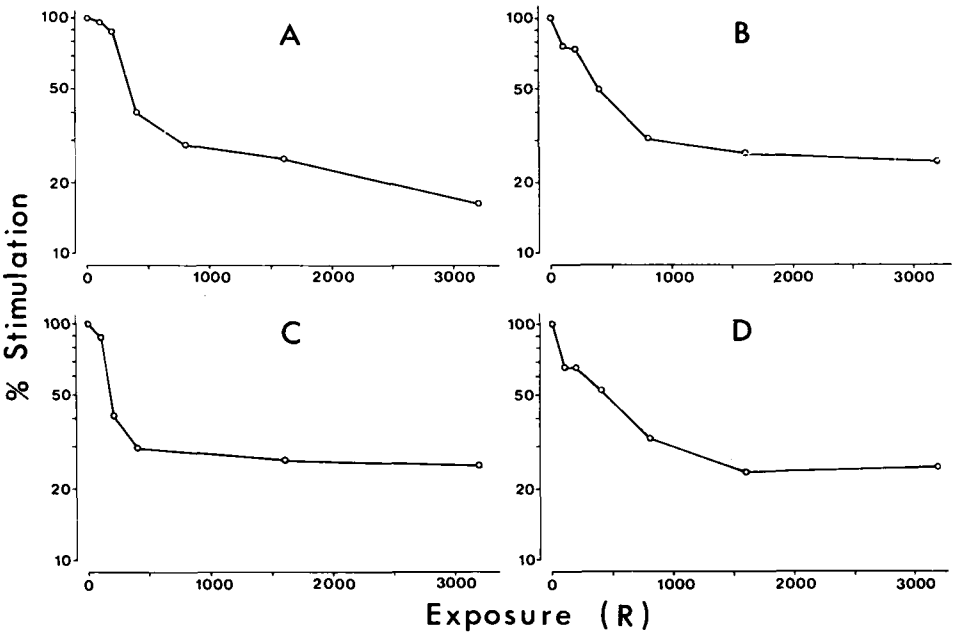

Fig. 1. Relative ^{3}H-thymidine uptakes of non-purified lymphocyte preparations incubated with 3 % of PHA after exposure to various radiation doses. Four different lymphocyte donors were employed.

exposed to 3 % of PHA 1 to 1½ hours after irradiation and their proliferative responses to this agent determined at day 4. Fig. 1 illustrates that there was a sharp reduction of ^{3}H-thymidine uptakes of the cultures by increasing the dose from 100 to 400 or 800 R. A further increase of irradiation dose only marginally decreased the PHA-response, thus creating a two-dose shaped curve. The results indicate that there are two populations of PHA-responsive lymphocytes; one which is relatively sensitive and one which is relatively resistant.

By determining the linear regression equation, $y = kx + 1$, for the values obtained in cell cultures yielding the plateaus of the curves, the proportion of 'resistant' PHA-responsive lymphocytes in the unirradiated cell preparations may be calculated. Table 1 shows the actual stimulations, expressed as cpm, of the non-irradiated mitogen stimulated cultures and the calculated proportions of the 'resistant' cell populations of the experiments are illustrated in Fig. 1.

Experiments were also conducted to determine the proliferative responses of irradiated non-purified lymphocytes to other concentrations of PHA as well as various concentrations of PWM and ConA (Fig. 2). These experiments yielded results analogous to those presented in Fig. 1; that is, a fairly linear decrease of stimulation occurred by increasing the dose within the dose range of 100 to 800 R whilst a further increase caused little change in ^{3}H-thymidine uptake. The absolute

Table 1

Absolute stimulations, expressed as CPM, of non-irradiated non-purified preparations of lymphocytes exposed to 3 per cent of PHA and their proportions of cells resistant to radiation. The relatives timulations after exposure to various radiation doses are illustrated in Fig. 1

Experiment*	^{3}H-thymidine incorporations (mean CPM)	Fraction of resistant cells** (per cent)	Radiation dose (R)
A	288 397	33.2	800–3 200
B	114 820	31.1	800–3 200
C	177 831	28.7	400–3 200
D	42 650	34.8	800–3 200

* The experiments refer to those presented in the diagrams of Fig. 1.
** The linear regression equations, $y = kx + 1$ of the relative PHA stimulations of cell preparations exposed to doses indicated within parenthesis were calculated (Fig. 1). The resistant fraction was determined by extrapolation to 0 R.

stimulations of the non-irradiated cultures and the size of the resistant cell populations are listed in Table 2.

Responsiveness of irradiated lymphocytes to antigens. The sensitivity of the responding cells of non-purified cell preparations involved in the MLC-reaction was also investigated. Fig. 3 presents the results of three such experiments using different donors of both responding and stimulating cells. The ^{3}H-thymidine uptake was markedly reduced by exposing the cells with doses up to 400 or 800 R.

Higher doses caused little or no further decrease of isotope incorporations. The stimulation of the non-irradiated allogeneic mixtures and the proportions of resistant MLC-reactive lymphocytes are listed in Table 3.

The responsiveness of irradiated non-purified lymphocytes to PPD was also tested after irradiation. Fig. 4 illustrates the results of two such experiments. The responsiveness of the cells to this antigen decreased in a fairly linear fashion with increasing doses. There was no tendency of the curves to plateau off at higher doses. In all tests the specific stimulation of cells exposed to 3 200 R was less than 1 per cent of that obtained in non-irradiated cultures. The absolute stimulations of non-irradiated PPD-exposed cultures are presented in Table 4.

Responsiveness of irradiated fractionated T- and B-lymphocytes to PHA. The results presented may indicate that polyclonal mitogens and allogeneic cells stimulate two populations of lymphocytes which differ with regard to sensitivity. Methods to separate

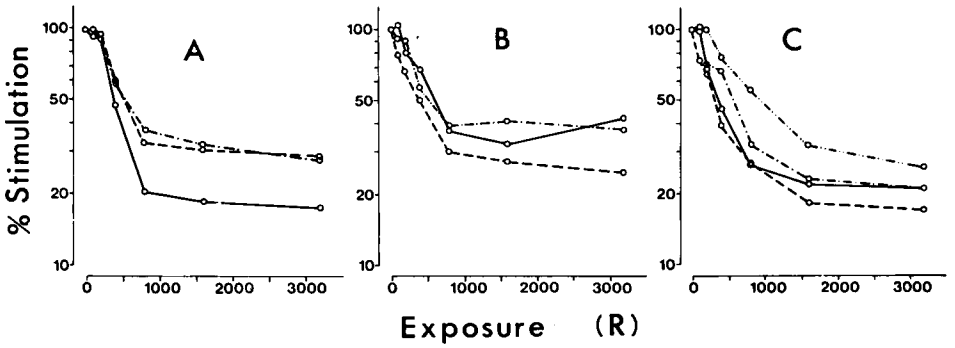

Fig. 2. Relative ^{3}H-thymidine uptakes of irradiated non-purified lymphocyte preparations exposed to various concentrations of three different phytomitogens. Left diagram: 1 % of PHA O—O, 0.33 % of PHA O---O, 0.11 % of PHA O-·-O. Middle diagram: 6 % of PWM O—O, 2 % of PWM O---O, 0.67 % of PWM O-·-O. Right diagram: 28 μg of ConA/ml O-··-O, 14 μg/ml O-·-O, 7 μg/ml O---O, 3.5 μg/ml O—O.

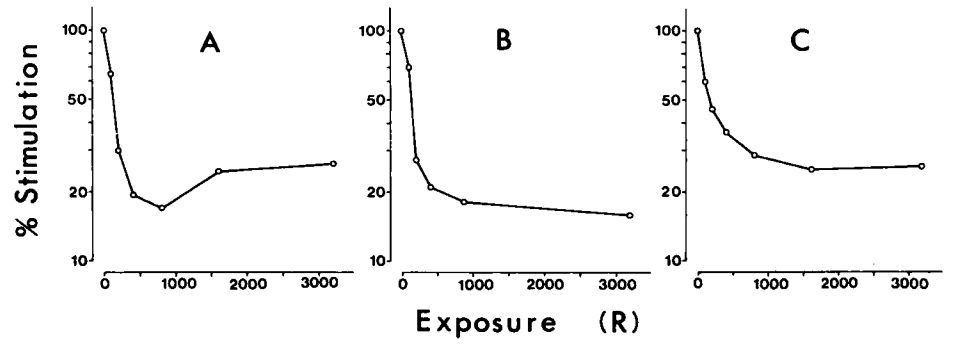

Fig. 3. Relative ^{3}H-thymidine incorporation of irradiated non-purified lymphocyte preparations cultured with allogeneic mitomycin treated lymphocytes. Three different responder-stimulator cell combinations are presented.

T- and B-cells exist, and it was thus examined whether the PHA-responses of cell preparations enriched for these cell types differ with regard to sensitivity.

Fig. 5. shows that the dose response profiles of irradiated non-purified, Fe-purified and T-cell preparations were similar. On the other hand the B-cell preparations differed since there was no sign of any resistant cell population.

Discussion

The present experiments were performed to investigate whether human lymphocytes which are responsive to different unspecific phytomitogens and antigens also differ with regard to sensitivity to irradiation. The results have shown that there is a relatively sharp reduction of the proliferative responses of the lymphocyte population

Table 2

Absolute stimulations of non-irradiated cells and proportions of resistant cells. These experiments are also presented in Fig. 2

Experiment	Mitogen-concentration	^{3}H-thymidine incorporations (mean CPM)	Fraction of resistant cells (per cent)	Radiation dose (R)
A	1 % PHA	165 400	21.2	800–3 200
	0.33 % PHA	145 100	34.2	800–3 200
	0.11 % PHA	125 600	40.5	800–3 200
B	6 % PWM	12 620	43.1	800–1 600
	2 % PWM	27 520	31.6	800–3 200
	0.67 % PWM	29 115	40.5	800–3 200
C	3.5 μg ConA/ml	65 810	23.3	1 600–3 200
	7.0 μg ConA/ml	89 515	19.9	1 600–3 200
	14.0 μg ConA/ml	76 015	24.6	1 600–3 200
	28.0 μg ConA/ml	52 070	38.9	1 600–3 200

Table 3

Absolute stimulations of non-irradiated cells and proportion of resistant cells of the experiments presented in Fig. 3

Experiment	^{3}H-thymidine incorporations (mean CPM)	Fraction of resistant cells (per cent)	Radiation dose (R)
A	144 750	22.7	1 600–3 200
B	89 130	20.4	400–3 200
C	40 570	28.7	800–3 200

to phytomitogens by exposing them to radiation doses within the range 100 to 800 R. However, a further increase of the dose has usually only a small influence on the responsiveness of the remaining lymphocytes to mitogens. The findings are in agreement with those of other authors who showed that irradiation of human lymphocytes with increasing doses in vitro reduces their responses to PHA in a two-dose-shaped fashion. This was found using both DNA-synthesis (CIRKOVIC 1969) and presence of blast cells (BRAEMAN & MOORE 1974) as parameters of reactivity of the irradiated cell cultures of mitogens. These results may indicate that there are two populations of mitogen responsive lymphocytes. One which is relatively sensitive and one which is relatively resistant. Two such lymphocyte populations have been observed in the thymus. The sensitive cells have been found to be anatomically located in the cortical

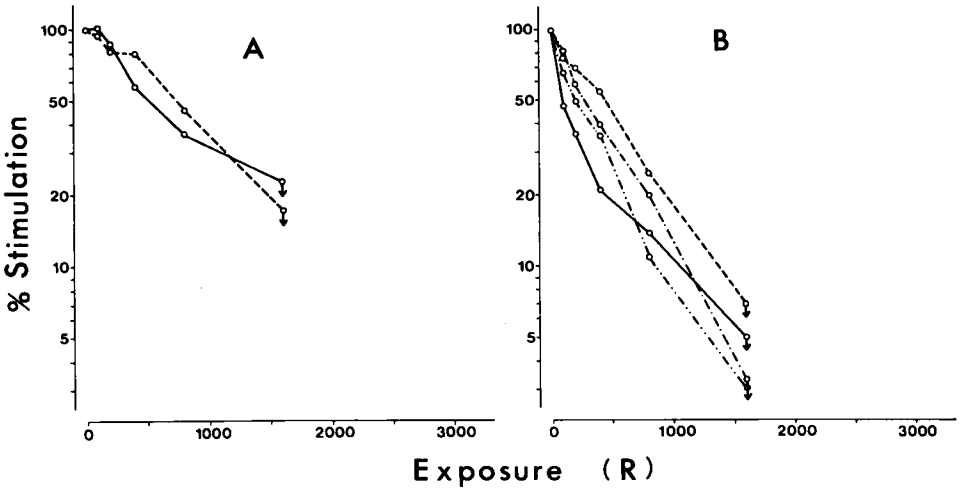

Fig. 4. Relative ^{3}H-thymidine uptakes of irradiated non-purified lymphocyte preparations exposed to various concentrations of PPD. Two different lymphocyte donors, selected for strong in vitro PPD-responses, were employed. 100 μg/ml o—o, 10 μg/ml o---o, 1.0 μg/ml o-·-o, 0.1 μg/ml o-··-o. Arrows indicate that the stimulations of cells exposed to 3 200 R were less then 0.5 %.

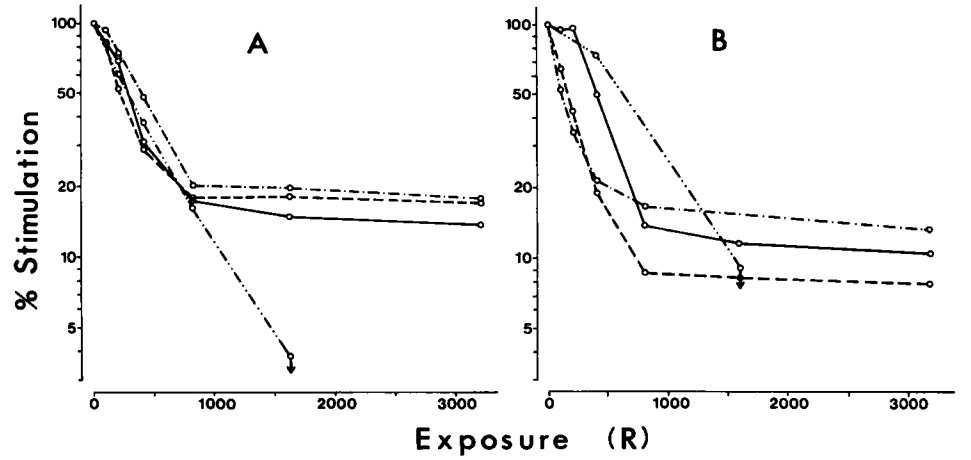

Fig. 5. Relative ^{3}H-thymidine uptakes of irradiated, fractionated lymphocytes incubated with 3 % of PHA. The diagram present the results of two separate experiments using different cell donors. Non-purified o—o, Fe-purified o---o, T-cells o-·-o, B-cells o-··-o. Arrows indicate that the stimulations of cells exposed to 3 200 R were less then 0.5 %.

parts of the organ and those which are more resistant in the thymic medulla (TROWELL 1961). These two cell populations also differ with regard to sensitivity to the lytic effect of adrenal corticosteroids (ISHIDATE & METCALF 1963) and immunologic competence (BLOMGREN & ANDERSSON 1968, 1971).

Table 4

Absolute stimulations of non-irradiated cells exposed to PPD. The relative stimulations of irradiated cells are presented in Fig. 4

Experiment	PPD-concentration (μg/ml)	^{3}H-thymidine incorporations (mean CPM)
A	100	4 519
	10	9 772
B	100	14 790
	10	21 800
	1.0	22 910
	0.1	19 055

Table 5

Absolute stimulations of non-irradiated cells and proportions of resistant cells of the various cell preparations exposed to 3 per cent of PHA presented in Fig. 5

Experiment	Cell preparation	^{3}H-thymidine incorporations (mean CPM)	Fraction of resistant cells (per cent)	Radiation dose (R)
A	Non-purified	101 950	18.1	800–3 200
	Fe-purified	85 110	17.9	800–3 200
	T-cells	83 445	21.3	800–3 200
	B-cells	13 975	—	
B	Non-purified	124 800	14.4	800–3 200
	Fe-purified	154 800	9.1	800–3 200
	T-cells	101 700	17.8	800–3 200
	B-cells	14 125	—	

In the present tests, a mitogenic stimulus was added 1 to 1½ hours after irradiation of the cells. Under these experimental conditions it was calculated that the resistant phytomitogen responsive sub-population of cells made up 10 to 40 per cent of the whole cell population. Other authors have presented data showing that the sensitivity of the cells may vary depending on when they are exposed to the mitogen. They become more sensitive as the time interval between exposure and addition of mitogen is increased (SCHREK & STEFANI 1964, VAUGHAN-SMITH & LING 1974). One explanation for this phenomenon is that phytomitogens trigger the lymphocytes to synthesize enzymes which are also involved in the repair of radiation injury. At present the possibility cannot be excluded that the potentially phytomitogen

responsive lymphocyte population is homogeneous with respect to sensitivity. However, the cell population may be heterogenous with regard to responsiveness to a phytomitogen. Those which are triggered to protein synthesis very soon after the mitogen has combined with its membranes associated receptor will survive. Lymphocytes which are activated late after the 'mitogen signal' will die because of a deficient repair system. The highly mitogen responsive lymphocytes could thus represent the resistant cell population. Another explanation could be that lymphocytes with a short generation time die after a short time period, whereas cells with a longer generation time survive for a longer time after irradiation.

There is evidence that phytomitogen lectins induce blast transformation of mainly T-cells in the human (JONDAL 1974).

However, several authors have found that B-cells are also blast transformed in such cultures (PHILLIPS & ROITT 1973, PHILLIPS & WEISROSE 1974, MELLSTEDT et coll. 1973). It is possible that human B-cells are unresponsive to phytomitogens but become transformed by soluble mediators released by phytomitogen activated T-cells. Such an observation has been made using Tetanus toxoid (GEHA & MERLER 1974) or PPD (BLOMGREN 1975) as a stimulatory agent. In the present investigation the PHA-responses of irradiated cell population enriched for T- and non-T-cells (mainly B-cells) were examined. It was observed that the dose response profiles of T-cells was similar to that of the original non-purified cell population. However, the B-cell preparations differed in the sense that there was no sign of the presence of any resistant cell population. One interpretation of these results is that non-T-lymphocytes are normally activated late after the addition of PHA. Thus, they will die in a dormant stage after irradiation, before having had time to build up any repair system.

Interestingly, the PPD-responsive lymphocyte population did not exhibit any detectable subpopulation of resistant cells. Evidently, this is not a specific trait of antigen reactive cell populations, since a resistant subpopulation could be sharply distinguished amongst MLC-responsive lymphocytes. It may be of relevance that the majority of the PPD-responsive lymphocytes have been presensitized to this antigen and the response in vitro may be regarded as a booster stimulation. This is not the case with lymphocytes which are responsive in the MLC.

In conclusion, by comparing the results of the present investigation with in vitro irradiation, with those of lymphocyte reactivity of patients having received external radiation therapy, certain points of agreement may be observed: (1) the phytomitogen responses of lymphocytes is not abolished even after massive doses of ionizing radiation; (2) tuberculin reactivity of lymphocytes disappears by relatively small doses of radiation.

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SUMMARY

Human lymphocytes were exposed to varying doses of roentgen irradiation in vitro and thereafter tested for reactivity to different polyclonal mitogens and antigens using DNA synthesis as a marker for viability. The dose response profiles obtained indicate that there are two subpopulations of lymphocytes which are responsive to phytohaemagglutinin, poke weed mitogen, concanavalin A and allogeneic cells. One is relatively sensitive to radiation and the other is relatively resistant. However, no 'resistant' PPD-tuberculin responsive cell population could be detected. Irradiated lymphocyte populations enriched for T-cells exhibited both a sensitive and a resistant PHA-responsive population, whereas cell populations enriched for B-cells only exhibited a radiation sensitive one.

ZUSAMMENFASSUNG

Menschliche Lymphozyten wurden in vitro unterschiedlichen Dosen von Röntgenstrahlen ausgesetzt und danach deren Reaktivität gegenüber verschiedenen polyclonalen Mitogenen und Antigenen unter Verwendung der DNS-Synthese als Kennzeichen für deren Viabilität geprüft. Die erhaltenen Dosisantwortprofile deuten darauf hin, dass zwei Subpopulationen von Lymphozyten vorhanden sind, welche auf Phytohämagglutinin, Mitogen von Nieswurz, Concanavalin A und allogene Zellen reagieren. Die eine ist gegenüber Strahlen relativ empfindlich, die andere relativ resistent. Es konnte jedoch keine „resistente" gegenüber PPD-Tuberculin empfindliche Zellpopulation entdeckt werden. Bestrahlte Lymphozytenpopulationen, in denen T-Zellen angereichert worden waren, zeigten sowohl eine sensitive als auch eine resistente PHA-empfindliche Population, während Zellpopulationen, in denen nur B-Lymphozyten angereichert worden waren, nur eine Strahlen-empfindliche Population zeigten.

RÉSUMÉ

Des lymphocytes humains ont été exposés à différentes doses de rayons de Roentgen in vitro; puis on a testé leur réactivité à divers mitogènes et antigènes polyclonaux en utilisant la synthèse de l'ADN comme indicateur de leur viabilité. Les profils de réponses aux doses obtenus indiquent qu'il y a deux sous-populations de lymphocytes qui répondent à la phytohémogglutinine, au mitogène d'héllebore, à la concanavaline A et aux cellules allogéniques. Une de ces sous-populations est relativement sensible aux radiations et l'autre relativement résistante. Cependant on n'a pas pu détecter de population cellulaire « résistante » et répondant à la tuberculine PPD. Les populations lymphocytaires irradiées enrichies en cellules T contiennent à la fois une population sensible et une population résistante répondant à la PHA, alors que les populations enrichies seulement en cellules B ont une population sensible aux radiations.

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