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BLOOD LYMPHOCYTES AFTER RADIATION THERAPY OF CARCINOMA OF PROSTATE AND URINARY BLADDER

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Local radiation therapy may result in a decreased number of lymphocytes in the peripheral blood. This side effect of the treatment has been extensively investigated in patients with mammary carcinoma receiving local irradiation (MEYER & SAYRE 1970, THOMAS et coll. 1971, MCCREDIE et coll. 1972, STJERNSWÄRD et coll. 1972, GLAS & WASSERMAN 1974, BLOMGREN et coll. 1974).

Two main types of lymphocytes have been recognized in vertebrates, including man, thymus-dependent cells, T cells, and cells dependent on the Bursa of Fabricius in birds and its possible analogue in mammals, B cells (for a review of T and B cells see ROITT et coll. 1969). Since the peripheral blood lymphocyte population represents a mixture of both T and B lymphocytes and possibly also other types of lymphocytes, attempts have been made to investigate whether the lymphopenia induced by local irradiation is due to a reduction of T or B or both cell types. Using appropriate in vitro markers for human T and B lymphocytes it was recently observed that B lymphocytes are reduced to a higher extent than T lymphocytes following local irradiation of patients with mammary carcinoma (BLOMGREN et coll.). In vitro responsiveness of the cells to phytohaemagglutinin (PHA) was much less affected than their response to purified protein derivate

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of tuberculin (PPD) which was strongly suppressed (GLAS & WASSERMAN, BLOMGREN et coll.).

The present investigation was undertaken to determine whether there is a similar change of the peripheral blood lymphocyte population following local irradiation of patients with bladder or prostatic carcinoma as in those with mammary carcinoma. This question is of interest since these two groups of patients receive radiation to completely different parts of the body. It may be mentioned that the thymus, in irradiated breast carcinoma, is partly or completely included within the irradiated field.

Material and Methods

The material consists of nineteen patients, 15 males and 4 females between 52 and 75 years old, attending Radiumhemmet for treatment of prostatic or urinary bladder carcinoma. All patients received external irradiation. Four patients were irradiated by a ^{60}Co unit with 100 cm source to skin distance, and fifteen patients by a 6 MV linear accelerator. The entire small pelvis was exposed to irradiation using two oblique anterior beams with wedge filters and a direct posterior beam. Each patient was dose-planned individually. Patients with urinary bladder carcinoma received various doses of irradiation depending on the stage of the tumour and whether surgery was planned after irradiation. Some patients received 3 600 rad, 12 $\times$ 300 rad in four weeks, some received 6 400 rad, 32 $\times$ 200 rad in eight weeks with two weeks of rest when half the dose was given, some received 8 400 rad, 3 $\times$ 100 rad daily $\times$ 28 in eight weeks with two weeks of rest in the middle of the treatment. Patients with prostatic carcinoma received a total tumor dose of 5 400 rad, 30 $\times$ 180 rad in six weeks.

Separation of peripheral blood leukocytes. Two methods were employed. (1) Venous blood was drawn in heparinized syringes. The lymphoid cells were separated by centrifugation on a Ficoll Isopaque gradient (Ficoll: Pharmacia Fine Chemicals, Inc. Stockholm, Sweden. Isopaque: Nyegaard, Oslo, Norway) as detailed by others (JONDAL et coll. 1972). The leukocyte suspensions were washed once by centrifugation, resuspended in Eagle's Minimal Essential Medium supplemented with Earle's salts (MEM) and counted in a Bürker chamber after crystal violet staining. These crude populations of lymphoid cells were used for lymphocyte stimulation tests and in the first set of tests were also used to examine the frequency of rosette forming cells. (2) Venous blood was collected in glass beakers without heparin. The blood was defibrinated by agitation of glass pearls present in the beakers. The leukocytes were separated from the erythrocytes by sedimentation at unit gravity after addition of a gelatin

Table 1

Number of lymphocytes and frequency of E and EAC rosette forming cells in the peripheral blood of patients with prostatic or urinary bladder carcinoma before and after irradiation. The frequency of rosette forming cells was established in nonpurified preparations of lymphoid cells. Ten patients were examined. The differences of the values obtained before and after were calculated for each patient separately. The mean values of these differences ± SE are presented. Probability values as calculated by Student's t-test are also indicated

	Mean ± SE		
	Before	After	Difference
Cell number per µl of blood	1802 ± 266	788 ± 159	−1021 ± 315 $p < 0.025$
EAC cells (%)	46.4 ± 6.9	24.3 ± 3.6	−22.3 ± 3.6 $p < 0.001$
E cells (%)	36.3 ± 7.3	42.6 ± 4.1	+6.3 ± 8.8 $p < 0.5$

solution as described in detail by COULSON & CHALMERS (1964). The leukocytes were then suspended in 10 ml of trisbuffered Hanks balanced salt solution supplemented with 10 % autologous serum and containing 0.4 g of iron powder. The tubes were then incubated at 37° C for 30 min and rotated every 5th minute. Phagocytic cells were then removed by a magnet (LUNDGREN et coll. 1968). This was followed by centrifugation through a layer of Ficoll Isopaque. Details of these procedures have been given before (BLOMGREN et coll.). These highly purified lymphocyte suspensions were used to examine the frequency of various rosette forming cells.

Rosette formation of lymphoid cells. Human T lymphocytes adhere sheep erythrocytes to their cell surface in vitro in an immunologically nonspecific way whilst B lymphocytes do not (BRAIN et coll. 1970, BRAIN & GORDON 1971, JONDAL et coll.). The latter cells on the other hand have membrane bound receptors for the activated complement factor C'3 (BIANCO et coll. 1970, MICHLMAYR & HUBER 1970, DUKOR et coll. 1971, JONDAL et coll.). The frequency of lymphoid cells forming rosettes with sheep erythrocytes (E cells) and those forming rosettes with sheep erythrocytes coated with specific rabbit antibodies and complement (EAC cells) were determined in crude lymphoid cell suspensions and in highly purified preparations of lymphocytes. The methods have recently been described (BLOMGREN et coll.).

Table 2

PHA response of lymphocytes from patients before and after irradiation. The PHA responses, expressed as cpm, are related to the mean responses of lymphocytes from two control subjects tested at the same time. Ten patients were tested. Their lymphocyte counts and frequency of E and EAC cells are presented in Table 1. The difference between the values obtained before and after irradiation are calculated as described in Table 1

Mean response in per cent of controls ± SE		
Before	After	Difference
85.1 ± 11.5	72.8 ± 11.4	−11.6 ± 14.8 $p > 0.5$

Lymphocyte stimulants. Phytohaemagglutinin (PHA, Bacto-phytohaemagglutinin M, Difco Lab., Detroit, Mich., USA). The contents of commercially available vials of PHA were dissolved in 5.0 ml of MEM. The cells were stimulated by PHA at a final concentration of 3.0 %. Purified protein derivate of tuberculin (PPD tuberculin, RT 23, Statens Seruminstitut, Copenhagen, Denmark).

Lymphocyte culture conditions. 0.5×10^6 lymphoid cells were cultured in screw cap glass tubes containing 1.0 ml of MEM with 10 % of human serum (HS) from AB, Rh neg. donors, 100 units of penicillin and 150 μg of streptomycin per ml. The HS was decomplemented by heating at 56° C for 30 min and stored at —20° C before use. The lymphocytes were stimulated by PHA or varying amounts of PPD. Control cultures received no stimulants. The cells were cultured for 5 days at 37° C in a humified, 5 % CO_2-air atmosphere. Twentyfour hours before termination of the cultures each tube received 0.1 ml of MEM containing 0.4 μCi of ^{14}C-thymidine (Radiochemical Center, Amersham, England. Specific activity 54 mCi/mM). The cultures were completed by centrifugation in the cold, washed twice in a balanced salt solution by centrifugation and precipitated twice in trichloroacetic acid. The precipitates were then dissolved in Soluene and the contents of the tubes transferred to vials containing scintillation fluid. Details of these procedures have been given by GLAS & WASSERMAN. Activity, expressed as counts per minute, cpm, was recorded by a Packard Scintillation Counter Model 3380.

General design of the tests. Lymphocytes were obtained from the patients within one week before beginning of irradiation and within three weeks after its completion and before any other kind of specific therapy was started. As

Table 3

Number of lymphocytes and frequency of E and EAC rosette forming cells in the peripheral blood of patients with prostatic or urinary bladder carcinoma before and after irradiation. The frequency of rosette forming cells was established in purified preparations of lymphoid cells. Eight patients were examined. The difference between the values obtained before and after irradiation are calculated as described in Table 1

	Mean ± SE		
	Before	After	Difference
Cell number per μl of blood	2085 ± 246	615 ± 110	−1470 ± 315 $p < 0.005$
EAC cells (%)	21.4 ± 15.9	15.9 ± 2.3	−5.4 ± 2.3 $p < 0.05$
E cells (%)	49.8 ± 2.9	51.6 ± 3.4	+1.4 ± 4.4 $p > 0.5$

discussed previously the degree of lymphocyte stimulation by nonspecific mitogens varies extensively from time to time although the determinations are performed using the same healthy cell donor and the experimental conditions are kept strictly constant (BLOMGREN et coll.). The reason for this inter-experimental variability is unknown, but it is probably not due to changes of the peripheral blood lymphocyte population of the cell donor. To overcome this variability the PHA responses of the lymphocytes from two healthy control persons were always tested every time a patient's lymphocytes were stimulated with the same mitogen. The lymphocytes from one healthy control were stimulated with various doses of PPD every time a patient's lymphocytes were exposed to this agent. The same control persons were used throughout this investigation.

All cultures were set up in duplicate. The background incorporations of ^{14}C-thymidine obtained in control cultures without stimulants were deduced from that obtained in PHA or PPD stimulated cultures. The degree of stimulation of the patient's lymphocytes were related to the mean stimulation obtained in the cultures from the respective control subject and expressed as per cent. The degree of stimulation, expressed as cpm of the lymphocytes from the control subject exposed to PHA, varied from 25 000 to 80 000. The PPD response varied fram 15 000 to 30 000.

The rosette forming tests are highly accurate in terms of frequency of E and EAC rosette forming cells in the peripheral blood lymphocyte population of the same healthy control subject tested at various times (BLOMGREN et coll.). To

Table 4

Frequency of E and EAC rosette forming cells in purified lymphocyte preparations from a control subject tested on three occasions at 2—3 month intervals. These tests were performed parallel with those presented in Table 3

	1	2	3
EAC cells (%)	14	14	15
E cells (%)	53	62	51

further strengthen this finding the lymphocytes from one control were tested parallel with those from the patients.

Results

PHA response and frequency of E and EAC rosette forming cells in nonpurified lymphoid cell populations. The peripheral blood lymphocyte population in 10 patients was first established with regard to cell number, cellular composition and relative PHA response. Three of these patients were treated for prostatic carcinoma and seven for urinary bladder carcinoma. Two received a total tumor dose of 8 400 rad, three 5 400 rad and five 3 600 rad. The results obtained from these patients are pooled.

The number of lymphocytes in the peripheral blood was reduced to less than 50 per cent after completion of irradiation (Table 1). In nonpurified lymphoid cell preparations the frequency of cells forming EAC rosettes was found to decrease significantly following irradiation whereas the E rosette forming cells exhibited a slight, but not significant relative increase; the relative response of the cells to PHA did not change significantly (Table 2).

PPD response and frequency of E and EAC rosette forming cells in purified lymphocyte populations. Nine patients were examined. Four of them were treated for prostatic carcinoma and five for bladder carcinoma. Four received a total tumor dose of 6 400 rad, four 5 400 rad and one 3 600 rad.

The number of lymphocytes in the peripheral blood was reduced to approximately 30 per cent following irradiation (Table 3). The frequency of EAC rosette forming cells in purified lymphocyte preparations was found to decrease significantly whilst the frequency of E rosette forming cells did not change markedly. Purified blood lymphocyte preparations from a healthy control were examined in parallel experiments with regard to frequency of E and EAC rosette

Table 5

PPD response of lymphocytes from patients before and after irradiation. The PPD responses, expressed as cpm, are related to the response of lymphocytes from one control subject tested at the same time. Seven atients were tested. Their lymphocyte counts and frequency of E and EAC cells are presented in Table 3. The differences between the values obtained before and after irradiation are calculated as described in Table 1. The value for PPD stimulation after irradiation for each patient was related to the value obtained before. The mean value of these determinations, expressed as per cent±SE, are presented

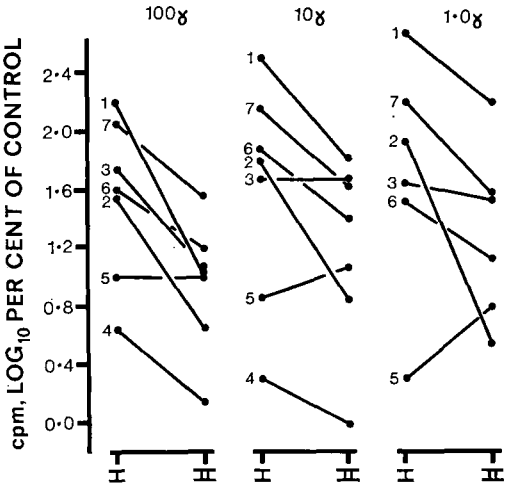
PPD concentration μg/ml	Mean±SE			Per cent of original value
	Before	After	Difference	
100	59.6±21.4	13.1±4.5	−46.5±19.6 $p < 0.1$	34.7±11.5 $p < 0.005$
10	93.8±41.7	28.8±9.1	−64.9±34.8 $p < 0.2$	59.8±21.9 $p < 0.2$
1.0	109.5±59.8	36.8±21.6	−72.8±39.5 $p < 0.2$	84.6±48.8 $p > 0.5$

forming cells. The frequency of these cells did not change markedly when tested at 2 to 3 month intervals (Table 4).

The response of lymphocytes to various concentrations of PPD in vitro was also tested before and after irradiation. The mean PPD responses of seven patients decreased following irradiation (Table 5). A statistically significant decrease was only observed when the cells were exposed to 100 μg of PPD. An extensive variability of the responses before irradiation occurred (Figure). It may be seen that there was a decrease of the PPD response in 6 out of 7 tested patients after irradiation. In general, lymphocytes from patients with a high PPD response before irradiation exhibited the strongest relative decrease afterwards.

Discussion

Several authors have stated that local irradiation of mammary carcinoma induces a lymphopenia in the peripheral blood (MEYER & SAYRE, THOMAS et coll., MCCREDIE et coll., STJERNSWÄRD et coll., GLAS & WASSERMAN, BLOMGREN et coll.). A similar lymphopenia seems to develop in patients receiving local irradiation to other parts of the body, although this is less extensively established (THOMAS et coll., MCCREDIE et coll.). Following local irradiation of breast carcinoma lymphocytes having receptors for $C^{1}3$, presumably mainly B lymphocytes, are reduced to a higher extent than lymphocytes binding sheep erythrocytes to their cell surface, T cells (BLOMGREN et coll.). No conclusive data



In vitro response of lymphocytes to various concentrations of PPD before (I) and after (II) local irradiation of the pelvic region. The responses are expressed as $\log_{10}$ per cent of the response of lymphocytes from a control subject. Seven patients were tested. Their values are illustrated separately and numbered from 1—7. The lymphocytes from patient 4 were not stimulated by 1.0 μg of PPD per ml and hence are not illustrated. Patients 3, 4, 5 and 6 received a total tumour dose of 6 400 rad and patients 1, 2 and 7 received 5 400 rad.

were obtained as to the response of the lymphocytes to PHA in vitro following irradiation, but the in vitro response of the cells to PPD decreased significantly (GLAS & WASSERMAN).

Two groups of patients were examined before and after radiation therapy. Both groups were heterogenous with regard to the age and sex of the patients, type of tumour and radiation dose delivered. It is evident that a significant lymphopenia developed in the peripheral blood of both groups of patients following irradiation. Counts of E and EAC rosette forming cells demonstrated that the frequency of the latter cells was reduced significantly whereas the proportion of E cells was not. The failure to demonstrate a significant increase of E rosette forming cells might have purely methodologic reasons, the reproducibility of the E rosette test being not so high as that of the EAC rosette test. In the first group of patients, the frequency of E and EAC rosette forming cells in nonpurified preparations of lymphoid cells was examined, in the second group highly purified lymphocyte suspensions were used. The results obtained from the latter tests probably only reflect changes of the frequency of T and B lymphocytes, whereas the results of the nonpurified are more difficult to interpret since human monocytes may also form EAC rosettes under the experimental conditions used and monocyte rosettes might sometimes be taken for lymphocyte rosettes (LAY & NUSSENZWEIG 1968). The present results in patients receiving irradiation to the pelvic region are in agreement with those obtained in patients with irradiated carcinoma of the breast. It is of interest that similar changes of the proportion of B and T cells seem to develop in the blood of patients receiving therapy with cytostatic drugs (SEN & BORELLA 1973).

Although the cellular composition of the lymphocyte population was observed to be significantly changed following irradiation, the relative response of the cells to PHA was not markedly changed. This is in agreement with previous reports that no evident change of PHA response could be observed after irradiation of breast carcinoma (BLOMGREN et coll.). Radiation induced decrease of the PHA response has been reported however (MILLARD 1965, THOMAS et coll., STJERNSWÄRD et coll.) whereas others have noticed an increase (MCCREDIE). Since there is evidence that PHA stimulates both B and T cells to increased DNA synthesis, at least when they are kept in a mixture (PHILLIPS & ROITT 1973), it is not surprising that a change of the T:B cell ratio does not result in any marked change of the PHA responsiveness.

The present results indicate that irradiation of the pelvic region decreases the response of the patient's lymphocytes to PPD in vitro. The type of human lymphocyte triggered by PPD is not known. In the mouse however there is strong evidence that low concentrations of PPD act as an antigenic stimulus for presensitized T cells, whereas higher concentrations activate B lymphocytes in a completely nonspecific fashion (SELZER & NILSSON 1972, NILSSON et coll. 1973). The results also indicate that the response of lymphocytes to both high and low concentrations of PPD decrease after pelvic irradiation. At present it is not possible to give any explanation for the impaired PPD response. However, the results are in agreement with those obtained in mammary carcinoma (GLAS & WASSERMAN).

In conclusion, local irradiation of the pelvic region induces the same types of changes of the peripheral blood lymphocyte population as local irradiation of the thoracic region, including the thymus, in mammary carcinoma.

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SUMMARY

The peripheral blood lymphocyte population in patients with prostatic and urinary bladder carcinoma was examined before and after local radiation therapy. The number of blood lymphocytes after therapy was reduced to 30 to 50 per cent. The frequency of lymphocytes bearing membrane bound receptors for activated complement, thymus-independent cells, decreased significantly whereas the frequency of lymphocytes binding sheep erythrocytes, thymus-dependent cells, remained essentially the same. The relative response of the lymphocytes to PHA was unchanged whereas their stimulation by PPD in vitro was significantly impaired following irradiation.

ZUSAMMENFASSUNG

Die periphere Blutlymphozyten Population von Patienten mit einem Prostata- oder Blasen-Karzinom wurde vor und nach lokaler Strahlentherapie untersucht. Die Zahl der Blutlymphozytes nach der Therapie war auf 30—50 % vermindert. Die Frequenz der Lymphozyten, die Membran-gebundene Rezeptoren für aktiviertes Komplement besitzen, — die Thymus-unabhängigen Zellen — viel signifikant, während die Frequenz der Schaferythrozyten bindenden Lymphozyten, — der Thymus-abhängiger Zellen —, im wesentlichen unverändert blieb. Die relative Antwort der Lymphozyten auf FHA war unverändert, während die Stimulation auf PPD in vitro nach Bestrahlung signifikant herabgesetzt war.

RÉSUMÉ

La population lymphocytaire du sang périphérique chez des malades atteints de cancer de la prostate et de la vessie a été examinée avant et après traitement local par les radiations. Le nombre des lymphocytes sanguins est réduit après le traitement de 30 à 50 %. La fréquence des lymphocytes portant sur leur membrane des récepteurs pour le complément activé, qui sont des cellules thymo-indépendantes, a diminué de façon significative alors que la fréquence des lymphocytes ayant des récepteurs pour les érythrocytes de mouton, qui sont des cellules thymo-dépendantes est restée dans l'ensemble la même. La réponse relative des lymphocytes à la PHA n'a pas été modifiée alors que leur stimulation par la PPD in vitro a été significativement diminuée après l'irradiation.

REFERENCES

Bianco C., Patrick R. and Nussenzweig V.: A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. I. Separation and characterization. J. exp. Med. 132 (1970), 702.

Blomgren H., Glas U., Melén B. and Wasserman J.: Blood lymphocytes after radiation therapy of mammary carcinoma. Acta radiol. Ther. Phys. Biol. 13 (1974), 185.

— — and Willetts W. A.: Rosette formation by peripheral lymphocytes. Clin. exp. Immunol. 8 (1970), 1.

Brain P. and Gordon J.: Rosette formation by peripheral lymphocytes. II. Inhibition of these phenomena. Clin. exp. Immunol. 8 (1971), 441.

Coulson A. S. and Chalmers G. G.: Separation of viable lymphocytes from human blood. Lancet 1 (1964), 468.

Dukor P., Bianco C. and Nussenzweig V.: Bone marrow origin of complement receptor lymphocytes. Europ. J. Immunol. 1 (1971), 491.

Glas U. and Wasserman J.: Effect of radiation treatment on cell-mediated immune response in carcinoma of the breast. Acta radiol. Ther. Phys. Biol. 13 (1974), 83.

Ilbery P. L. T., Rickenson A. B. and Thrum C. E.: Blood lymphocyte replicating ability as a measurement of radiation dosage. Brit. J. Radiol. 44 (1971), 834.

Jondal M., Holm G. and Wigzell H.: Surface markers on human T and B lymphocytes. J. exp. Med. 136 (1972), 207.

Lay W. H. and Nussenzweig V.: Receptors for complement on leukocytes. J. exp. Med. 128 (1968), 991.

LUNDGREN G., ZUKOSKI Ch. F. and MÖLLER G.: Differential effects of human granulocytes and lymphocytes on human fibroblasts in vitro. Clin. exp. Immunol. 3 (1968), 817.

MCCREDIE J. A., INCH W. R. and SUTHERLAND R. M.: Effect of postoperative radiotherapy on peripheral blood lymphocytes in patients with carcinoma of the breast. Cancer 29 (1972), 349.

MEYER K. K. and SAYRE P.: Radiation-induced lymphocyte-immune deficiency. Arch. Surg. 101 (1970), 114.

MICHILMAYR G. and HUBER H.: Receptor sites for complement on certain human peripheral blood lymphocytes. J. Immunol. 105 (1970), 670.

MILLARD R. E.: Effect of previous irradiation on the transformation of blood lymphocytes. J. clin. Pathol. 18 (1965), 783.

NILSSON B. S., SULTZER B. M. and BULLOCK W. W.: Purified protein derivate of tuberculin induces immunoglobulin production in normal mouse spleen cells. J. exp. Med. 137 (1973), 127.

PHILLIPS B. and ROITT I. M.: Evidence for transformation of human B lymphocytes by PHA. Nature New Biology 241 (1973), 254.

ROITT I. M., GREAVES M. F., TORRIGLANTI G., BROSTOFF J. and PLAYFAIR J. H.: The cellular basis of immunological responses. Lancet 2 (1969), 367.

SELZER P. M. and NILSSON B. S.: PPD tuberculin acts as a mitogen for bone derived lymphocytes only. Nature New Biology 240 (1972), 198.

SEN I. and BORELLA L.: Expression of cell surface markers on T and B lymphocytes after long-term chemotherapy of acute leukemia. Cell. Immunol. 9 (1973), 84.

STJERNSWÄRD J., JONDAL M., VANKY F., WIGZELL H. and SEALY R.: Lymphopenia and change in distribution of human B and T lymphocytes in peripheral blood induced by irradiation for mammary carcinoma. Lancet 24 (1972), 1352.

THOMAS J. M., COY P., LEWIS H. S. and YUEN A.: Effect of therapeutic irradiation on lymphocyte transformation in lung cancer. Cancer 27 (1971), 1046.