

# A Comparison of the Acute Effects of Radiation Therapy, Including or Excluding the Thymus, on the Lymphocyte Subpopulations of Cancer Patients

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**ABSTRACT** Radiation therapy to either mediastinum or pelvis causes a rapid decrease in circulating lymphocytes of both B and T types and in addition an impairment in the function of the remaining lymphocytes, as measured by their ability to proliferate in response to mitogens. The acute depression is short-lived. Substantial recovery is apparent within 3 wk after cessation of therapy; however, most patients show a modest, chronic depression in both numbers and functional capacities of circulating lymphocytes. T cells are somewhat more sensitive than B cells, but both are affected.

Irradiation of the thymus per se seems to have little influence on the acute changes which occur, as patients receiving pelvic and mediastinal (including thymic) radiotherapy show a similar degree of lymphopenia and depression of lymphocyte responsiveness.

## INTRODUCTION

In experimental situations in vitro, thymus-derived (T) lymphocytes and antibodies (the product of bone marrow-derived lymphocytes), in conjunction with "non-T" cells or complement, can destroy tumor cells (1-4). In virtually every case of clinical cancer, these mechanisms are inadequate to eradicate an established tumor, as "spontaneous" regressions are very rare. Nevertheless, it is possible that the degree of competence of a patient's immune system may be an important factor in the clinical course of his disease. For this reason, it is important to examine (a) the immunosuppressive effects

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of various modalities of cancer therapy, and (b) the correlation, if any, of such immunosuppression with prognosis.

In this study, we have examined prospectively the effects of radiation therapy (RT)<sup>1</sup> delivered to the mediastinal area, including the thymus, on the numbers and functional capacities of certain subpopulations of circulating lymphocytes. In particular, we have tried to test the hypothesis that irradiation of the thymus per se can decrease the numbers and impair the function of circulating T lymphocytes (5). Patients receiving pelvic irradiation serve as a good control group because they receive a similar dose of radiation to tissue and blood without thymic irradiation. Patients were evaluated before, during, and after therapy.

To identify the subclasses of lymphocytes, we used three methods: rosette formation, membrane immunofluorescence (MF), and blastogenic response to mitogens. Approximately 60% of human peripheral blood lymphocytes (PBL) and 95% of human thymocytes form spontaneous rosettes with sheep red blood cells (E

<sup>1</sup> *Abbreviations used in this paper:* A, rabbit hemolysin; C, human complement; CRL, complement-receptor lymphocytes; E, erythrocytes; EHT, equine antihuman thymus globulin; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HuABS, human serum AB; IMF, indirect membrane immunofluorescence; MF, membrane immunofluorescence; NSABP, National Surgical Adjuvant Breast Protocol; PBL, peripheral blood lymphocytes; PHA-P, phytohemagglutinin; PWM, pokeweed mitogen powder; RFC, rosette-forming cells; RHB, rabbit antihuman brain serum; RHL, rabbit antihuman light chain serum; RT, radiotherapy; SIg, surface immunoglobulin; SRBC, sheep red blood cells.

TABLE I  
*Diagnosis, Stage of Disease, and Radiation Treatment of the 28 Patients Reported*

Group	Diagnosis	Previous surgery	Number of patients, stage of disease	External radiation dose rate	Total external radiation	Radium implant dose rate	Thymic radiation
				<i>rads/wk</i>	<i>rads</i>	<i>mg-h</i>	<i>rads</i>
D	Cervical carcinoma	No	1, stage I 2, stage II	800-1,000	2@5,000 2@3,000-4,000	1@2,000-3,000 3@6,000-7,000	None
	Ovarian carcinoma	Yes	1, stage IV 2, localized	800-1,000	4,000-5,000	None	None
	Endometrial carcinoma	No	1, regional One patient each stage II, III, IV	800-1,000	5,000-6,000	None	None
B	Breast carcinoma	Yes	9, localized 6, regional 1, systemic	2@600-800 14@800-1,000	4,000-5,000	None	4,000-5,000
	Lung carcinoma	No	1, systemic	1,200-1,500	4,000	None	4,000
	Esophageal carcinoma	No	1, regional	800-1,000	6,000	None	6,000

rosettes). They are thought to represent human T lymphocytes, both mature and immature (6, 7). E rosettes prepared by another method have been said to correlate with cell-mediated immune competence (8).

Approximately 30% of human peripheral blood mononuclear cells have been shown to have receptors for activated complement on their surface. Most of the complement-receptor lymphocytes (CRL) belong to the antibody-forming or B cell line, while a few are monocytes (9-11). To demonstrate CRL ("B cells"), EAC rosettes are made with sheep erythrocytes (E) coated with a nonagglutinating concentration of rabbit hemolysin (A) and human complement (C) (9, 12).

T and B cells can also be distinguished by their membrane-associated antigens. T cells have, on their surface, antigen(s) cross-reactive with brain tissue both in mice (13) and in man (14, 15). B cells have surface immunoglobulin easily detectable by immunofluorescence (16, 17). We have used direct and indirect MF to enumerate cells bearing on their surface either immunoglobulin (most B cells) or an antigen cross-reactive with brain (T cells). In addition, we have used a human autoantibody to identify T cells (18).

### METHODS

The 21 patients receiving radiation therapy in this study were treated by the Varian 6 meV accelerator, half value layer 18 mm Cu at a target to skin distance of 100 cm (Varian Associates, Palo Alto, Calif.). All patients were treated with five fractions per week. Treatment ports for patients with breast carcinoma were planned in accordance with the National Surgical Adjuvant Breast Protocol (NSABP) study 4, which compares radical mastectomy with total (simple) mastectomy with or without RT as therapy for primary stage I and II cancers. This plan includes irradiation of the chest wall, internal mammary, and supraclavicular and axillary lymphatics, after simple mastectomy. The entire esophagus of the one patient with carcinoma of the esophagus was treated with one anterior and two posterior oblique fields. The mediastinum and perihilar

regions of the patient with lung carcinoma were treated with both anterior and posterior fields. The entire pelvis of each patient receiving therapy for pelvic malignancy was treated with 15 x 15-cm anterior and posterior opposed ports. Three of the pelvic patients receiving radium treatments were treated with the Fletcher-Suit afterloading applicator, and one patient received a vaginal radium mold.

The subjects studied were classified into four groups: group A, 21 normal subjects; group B, 18 patients receiving prophylactic RT for cancer in the mediastinal area (in most cases for breast cancer); group C, 8 patients who had mastectomies but no RT for breast cancer; and group D, 10 patients receiving RT to the pelvic area (cervical, ovarian, and endometrial cancer). Since patients were studied before, as well as during and after RT, in effect each subject served as his own control. The normal subjects were laboratory and secretarial personnel. The patients in group B had most of the thymus irradiated during RT. The patients in group C and 10 of 18 in group B were part of the NSABP study and they can be considered comparable before treatment, since the allocation into groups was randomly done; the remaining patients in group B had more advanced cancer. See Table I for a detailed description of the patients in groups B and D.

*Antisera.* Rabbit antihuman brain serum (RHB) was made by immunizing New Zealand white rabbits with normal human brain obtained at autopsy. The tissue was washed and homogenized, and rabbits were injected with 100 mg brain homogenate emulsified in Freund's complete adjuvant into the hind foot pads on day 0, plus subcutaneous injections of 100 mg brain homogenate in Hanks' balanced salt solution (HBSS; Grand Island Biological Co. [GIBCO], Oakland, Calif.) the next day, 1 g on day 12, and 100 mg on days 19 and 29; then, they were bled on day 35. Rabbit antihuman light chain serum (RHL) was prepared by immunizing New Zealand white rabbits with the light chains of human immunoglobulin prepared from pooled human AB sera (GIBCO) (19). The immunization schedule was the same as above but the rabbits received 1 mg/injection. Equine antihuman thymus globulin (EHT: Lot 16,138-9) was provided courtesy of Dr. Gary D. Gray, Upjohn Co., Kalamazoo, Mich. A naturally occurring human IgM antibody (Th), which reacts specifically with human thymus-derived lymphocytes (18), was the gift of Dr. D. B. Thomas of the National Institute for Medical Research, Mill Hill, London.

RHB and RHL were absorbed without dilution, while EHT was diluted 1:50 in HBSS. 10 ml serum was absorbed repeatedly with  $10^8$  washed, packed lymphocytes at room temperature for 1 h and then in an ice bath for another hour.

**Lymphocyte isolation.** Human PBL were isolated from heparinized blood by isopycnic density gradient centrifugation (20) at room temperature. The blood was diluted threefold with NaCl-EDTA (pH 7.4, 0.85% NaCl, 0.05% EDTA) and carefully layered over a solution of 10 parts Hypaque (38% wt/vol; sodium diatrizoate, Winthrop Laboratories, New York) and 24 parts Ficoll (9% wt/vol; refractive index 1.3468; Pharmacia, Uppsala, Sweden).

After centrifugation in a swinging bucket rotor at 275 *g* for 35 min, the cells layered at the interface were aspirated and centrifuged for 15 min at 800 *g*. The cells, comprising mainly lymphocytes, were washed three times and resuspended to a final concentration of  $5 \times 10^6$  mononuclear cells/ml in HBSS. The usual yield of lymphocytes from normal subjects is  $1-2 \times 10^6$ /ml of whole blood, giving an efficiency of 80-90%. Isolation of lymphocytes late in RT is less satisfactory, both with respect to yield (30-70% of available lymphocytes) and purity (5-50% contamination with polymorphonuclear leukocytes).

**Lymphocyte cultures.** All media were at pH 7.4, supplemented with 10,000 IU of penicillin (potassium penicillin G, E. R. Squibb and Sons, Princeton, N. J.), 25  $\mu$ g Fungizone (amphotericin B, GIBCO), 3  $\mu$ g gentamicin (gentamicin sulfate; Schering Diagnostics, Port Redding, N. J.), and 10 mg streptomycin (streptomycin sulfate; Pfizer Laboratories Division, Pfizer Inc., New York) per 100 ml.

HBSS without bicarbonate and Roswell Park Memorial Institute Medium 1640 containing 25 mM HEPES buffer (RPMI-1640) were purchased from GIBCO. RPMI-1640 was supplemented with 2 mM glutamine (GIBCO). Fetal bovine serum (FBS) and human serum AB (HuABS) obtained from GIBCO were heated to 56° for 45 min before use.

Lymphocytes were cultured for 5 days in RPMI-1640 supplemented with 5% HuABS in plastic microculture plates (Microbiological Associates, Bethesda, Md.) at 37°C with the appropriate concentration of mitogen. Each concentration of mitogen was added to six wells; the lymphocyte preparations were also cultured without mitogen. The cultures contained  $1 \times 10^6$  mononuclear cells/well in 0.15-0.20 ml, final volume. Purified phytohemagglutinin-P (PHA-P; Wellcome Reagents, Ltd., Beckenham, Kent) was used at the concentrations of 0.1, 0.5, and 2.5  $\mu$ g/well. Pokeweed mitogen powder (PWM; GIBCO) was diluted with HBSS and approximately 0.5 and 2.5  $\mu$ g used/well. After 4 days of culture, 0.1 ml of fresh RPMI-1640 containing 20% FBS and 0.4  $\mu$ Ci [ $^3$ H]thymidine ([ $^3$ H]TdR; [*methyl*- $^3$ H]thymidine, TRK 120, sp act 19 Ci/mM, Amersham/Searle Corp., Arlington Heights, Ill.) was added. The samples were harvested 24 h later with a multiple automatic sample harvesting unit (Microbiological Associates) onto glass fiber filter paper, which was washed extensively with 5% trichloroacetic acid. The dried filter paper was placed in a scintillation vial with 10 ml of 4% Permafluor (Packard Instrument Co., Inc., Downers Grove, Ill.) in toluene and the radioactivity incorporated was determined by scintillation counting.

**Rosettes.** To form spontaneous rosettes (total erythrocyte-rosette-forming cells [E-RFC] = T cells),  $0.5-1 \times 10^6$  mononuclear cells in 0.25 ml HBSS were mixed with 0.25 ml of 1% washed sheep red blood cells (SRBC; Mission Laboratories, Rosemead, Calif.) in a polypropylene test tube

(Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), incubated in a 37°C water bath for 15 min, centrifuged at 200 *g* for 5 min and immediately placed in an ice water bath. The next morning (18-22 h later), the mixture was gently resuspended by tilting the tube between thumb and forefinger (21), and the E-RFC were counted. Note that the ratio of SRBC to mononuclear cells is very high (>100:1), the suspension is incubated in HBSS at 37°C, and the rosettes are kept cold overnight. This gives stable rosettes, and nearly all normal subjects' T-cells form E-rosettes.

"Active" E-RFC (a T-cell subpopulation?) were prepared by the method of Wybran, Carr, and Fudenberg (22). To wit,  $0.5 \times 10^6$  mononuclear cells in 0.1 ml HBSS and 0.1 ml sterile FBS (absorbed with SRBC and agarose) were mixed and incubated at 37° for 1 h. SRBC were added (eight per lymphocyte in 0.1 ml HBSS) and the suspension centrifuged at 200 *g* for 5 min. The rosettes were immediately gently resuspended and counted.

Complement-coated erythrocytes (EAC) were prepared by incubating 5% thrice-washed SRBC (E) with rabbit antiserum hemolysin (A) 1:2,000 (GIBCO) in a 37°C water bath for 30 min. The antibody-coated cells (EA) were washed three times and incubated (immediately or after 1-4 days' refrigeration) at 37° for 30 min with 1:40 human complement (C; pooled, fresh-frozen normal human serum). The EAC were washed three times and resuspended to 1% in HBSS. To enumerate cells with receptors for C3 (B cells and monocytes),  $0.5-1.0 \times 10^6$  mononuclear cells in 0.25 ml HBSS were mixed with 0.25 ml 1% EAC-SRBC. The tubes were immediately spun at 200 *g* for 2 min and allowed to stand at room temperature for 15-30 min. The mixture was resuspended vigorously (by brief but vigorous agitation with a vortex mixer to destroy any spontaneous E-RFC which had formed) and counted immediately. Monocyte contamination was estimated from Wright's-stained smears at  $6.4 \pm 1.3\%$  of total mononuclear cells.

The number of RFC was determined by both a "fresh" count and (for E and EAC rosettes) a fixed slide count. The fresh counts were performed with the aid of a vital stain. One drop of a 1:2 dilution of 1% toluidine blue (23) in methanol, previously dried on the bottom of a 12  $\times$  75-mm glass tube, was redissolved in 0.1 ml of saline; 0.2 ml of the RFC suspension was carefully added, and within 1-3 min, the suspension was gently mixed, a portion placed on a hemocytometer, and an area of 4 mm<sup>2</sup> scored. Mononuclear cells were scored "+" (four or more SRBC adherent) or "-" (three or fewer adherent SRBC); polymorphonuclear leukocytes or cells with granules were ignored. For active E-RFC, our criterion was three red blood cells attached (cf. Wybran's criterion of one attached erythrocyte; ref. 22). The number of RFC per milliliter of peripheral blood is determined from the percent positive cells and the number of mononuclear cells present per milliliter of whole blood.

Fixed slide preparations were made by first pipetting 0.1 ml of the freshly resuspended RFC into 0.2 ml 1% glutaraldehyde in Tyrode's solution. Duplicate slides were prepared, with the addition of a small drop of FBS (absorbed with SRBC). After drying, the slides were stained with Wright's or May-Grünwald stain. At least 200 lymphocytes were scored per slide; three attached SRBC was considered positive. The fixed slides have the advantage of permanence and allow better examination of the central cells of the rosette.

**Membrane immunofluorescence.** For direct membrane immunofluorescence (MF) (B cells), 10  $\mu$ l fluorescein-labeled goat polyvalent antiserum to human immunoglobulins (Meloy Laboratories Inc., Springfield, Va., C201), were

TABLE II  
Reagents for Indirect Membrane Fluorescence

Serum*	Dilution	Fluoresceinated serum	Dilution	Source
Equine anti-human thymus globulin (EHT)	1:1,000-1:10,000	Rabbit anti-equine gamma globulin	1:20	Miles-Yeda
Rabbit anti-human brain (RHB)	1:100	Goat anti-rabbit gamma globulin	1:20	Meloy
Rabbit anti-human L chains (RHL)	1:100	Goat anti-rabbit gamma globulin	1:20	Meloy
Human IgM autoantibody ('Th')	1:800	Goat anti-human IgM	1:20	Miles-Yeda

\* See text for source or preparation.

mixed with  $1.5 \times 10^6$  lymphocytes in 0.2 ml HBSS, incubated for 1 h, washed thrice in HBSS in a refrigerated centrifuge (200 *g* for 5 min), and resuspended in glycerine mounting medium (Becton-Dickinson & Co., Rutherford, N. J.). All procedures and solutions were at 4°C. The number of mononuclear cells displaying MF of a "ring" or granular type was determined with a Nikon SIR-F fluorescence microscope (Nikon Inc., Instrument Group, EPOI, Garden City, N. Y.); total mononuclear cells in the same fields were counted with visible light.

For indirect membrane fluorescence (IMF) tests (T or B cells) the lymphocytes were incubated with the antiserum diluted in HBSS for 1 h, washed as above, and resuspended in 0.2-0.3 ml of appropriately diluted fluoresceinated antiserum (see Table II). They were incubated, washed, resuspended, and counted as above.

## RESULTS

Four groups of subjects were initially studied: group A, normal laboratory and secretarial personnel; group B, patients with breast cancer who received mediastinal RT; group C, patients with breast cancer who did not receive RT; and group D, patients who received pelvic RT. In addition, one patient with esophageal and one

with lung cancer who showed results like those of the other mediastinal RT patients were included in group B. Groups B and D were followed during and after RT. 16 patients (including 10 on the NSABP study) receiving prophylactic RT for breast cancer were studied. The breast cancer controls (group C) were all on the NSABP protocol study 4, which compares radical mastectomy and simple mastectomy with or without RT in primary stage I and II tumors. The patients receiving pelvic RT comprised four cervical, three ovarian, and three endometrial carcinomas (see Table I).

Table III compares various parameters of the four groups before RT. There were few significant differences between the groups. The normal subjects were younger, on average, than the patients. The patients designated to receive RT for mammary carcinoma had significantly lower numbers of total E rosettes and active E rosettes than either normal subjects or patients about to receive pelvic RT. They were not significantly different, however, from the breast cancer patients who did not receive RT.

TABLE III  
Comparison of Normal and Patient Groups with Respect to Age and to Numbers and Composition of Leukocytes in the Blood

	Group A, normal	Group B, mediastinal RT (pretreatment)	Group C, breast cancer controls	Group D, pelvic RT (pretreatment)
Age, yr	32.5 ±2.0	50.2§ ±3.6	54.5§ ±3.7	49.0‡ ±4.2
Total E-RFC, cells/ml $\times 10^{-5}$ *	16.8 ±1.6	12.1‡ ±1.1	12.4 ±3.4	16.6   ±1.8
EAC-RFC, cells/ml $\times 10^{-5}$ *	7.0 ±0.7	5.3 ±1.9	5.4 ±1.1	6.7 ±0.9
Active E-RFC, cells/ml $\times 10^{-5}$ *	6.3 ±0.7	3.0§ ±0.8	5.9 ±1.7	6.6   ±1.3
Mononuclear cells, cells/ml $\times 10^{-5}$ *	31.0 ±3.0	24.0 ±2.0	24.0 ±4.0	29.2 ±2.5
WBC, cells/ml $\times 10^{-5}$ *	73.0 ±4.0	86.0 ±10.0	87.0 ±16.0	96.0 ±14.0

\* Mean  $\pm$  SEM.

‡ Compared to group A,  $P < 0.05$  by Student's *t* test.

§ Compared to group A,  $P < 0.01$  by Student's *t* test.

|| Compared to group B,  $P < 0.05$  by Student's *t* test.

TABLE IV  
Precision of the Rosette Tests

	Rosettes	
	E-RFC	EAC-RFC
%		
Repeated sampling of normal subjects on different days†		
K. T. Fresh	56.4±1.6 (8)*	23.8±1.7 (6)
Slides	67.6±1.2 (6)	24.5±2.2 (3)
P. B. Fresh	59.2±4.9 (8)	25.9±2.1 (16)
Slides	72.4±3.0 (13)	22.4±1.9 (6)
Replicate samples: same subject, same day‡		
Exp. 1 Mean±SE	64.5±1.8	25.4±1.2
Range	57.3-70.9	22.0-28.7
Exp. 2 Mean±SE	59.5±2.0	20.9±1.3
Range	51.6-65.1	17.5-24.9

\* Mean±SEM (number of tests). Duplicate samples were prepared on each day, and the mean of the average values from *n* days was determined.

† Rosettes were counted either by placing them directly into a hemocytometer with toluidine blue (fresh) or after fixation with glutaraldehyde and smearing and staining with Wright's stain (slides).

‡ Each experiment represents sextuplicate E and EAC samples on one subject, prepared simultaneously.

*Comparison of rosette and immunofluorescence methods in normal subjects.* Table IV documents the reproducibility of the RFC tests (whether fresh counts or fixed slide counts) obtained at different times, over a 6-mo period, on the same subjects. On a few occasions, to determine the precision of our methods, rosette tests were run with many replicate samples at once (Table IV). The reproducibility of all tests was satisfactory.

Table V illustrates the correlation between immunofluorescent assays for surface immunoglobulin (SIg) on

TABLE V  
Correlation between Rosette and Immunofluorescence Assays for Subpopulations of Human Lymphocytes

Tests	Immunofluorescence	Rosettes	
T cell	T cell antigens (indirect tests)	Spontaneous E	
	Autoantibody (Th)‡ 13.8±1.0 (35)*		16.8±1.6 (21)*
	Anti-brain (RHB)‡ 15.4±1.4 (26)*		
B cell	Surface Ig	EAC	
	Direct (GHIg)‡ 7.2±0.5 (35)*		7.8±0.7 (21)*
	Indirect (RHL)‡ 6.5±1.4 (5)*		

\* Number of positive cells × 10<sup>6</sup>/ml of blood. Mean±SEM (number of samples).

† GHIg (fluorescein-conjugated goat anti-human immunoglobulins); RHL (rabbit anti-human light chains); RHB (rabbit anti-human brain); Th (a human IgM autoantibody). RHL and RHB were absorbed with human RBC, sheep RBC, and agarose; see text for methods.

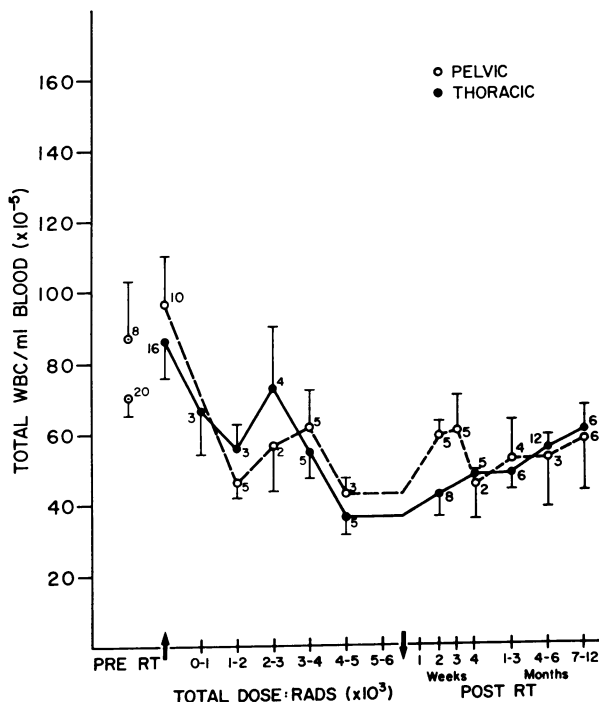


FIGURE 1 The effect of RT on the total leukocyte count. ○---○, 10 patients receiving pelvic irradiation (group D); ●—●, 16 patients receiving mediastinal irradiation (group B), sampled before, during, and after RT; ○, 8 breast cancer controls (group C) and 20 normal subjects (group A) are included for comparison. Means±SEM of *n* determinations.

B cells and EAC-RFC, and between immunofluorescence assays for T cells and E-RFC. The spontaneous rosette test (E-RFC), which measures most or all T cells, gave an average value for 21 normal subjects of 16.8 × 10<sup>6</sup>/ml of blood. This was in good agreement with two IMF tests for T cells; Th, a human autoantibody specific for T lymphocytes (18) and RHB, a rabbit antiserum to human brain, which cross-reacts with T lymphocytes (J. A. Stratton and P. E. Byfield, in preparation). This represents about 50-60% of all circulating mononuclear cells. EHT stained virtually all lymphocytes very brightly, and the staining of B cells could not be eliminated selectively either by dilution of EHT or by 18 absorptions with 10<sup>7</sup> chronic lymphocytic leukemic cells (B cells) per milliliter per absorption.

To identify B cells, the direct MF test for SIg and an indirect membrane fluorescence test (RHL), used to detect SIg, stained similar numbers of cells (Table V). Since the direct test was equivalent to the indirect, but simpler, it was used for the remaining studies. These values represent 20-30% of circulating mononuclear cells. The EAC rosette test, which measures cells bearing C3 receptors, gave a slightly higher value

for B cells than MF, perhaps because monocytes bear C3 receptors but not SIg. In a patient with Bruton's agammaglobulinemia, we found no cells with SIg in the circulation, but normal numbers of E- and EAC-RFC (24).

*Isolation of lymphocyte subpopulations.* The yield and purity of the mononuclear cell preparations from blood of normal subjects was superior to that from patients' blood, even before RT. Contamination by polymorphonuclear leukocytes averaged 10% in normals and was significantly greater in patients both before ( $P < 0.05$ ) and after ( $P < 0.01$ ) RT. There was no correlation between the percent recovery and contamination with polymorphonuclear leukocytes. Greaves has stated that the ratio of recovered T and B lymphocytes is related to the percent recovery of lymphocytes (UCLA Workshop, Cell Surface Markers for Human Lymphocyte Populations, April 1974). We have confirmed this correlation in normal subjects ( $r = 0.584$ ,  $P < 0.05$ ) but found no such correlation in the patient groups either before or after irradiation.

The ratio of T cells to B cells of normal subjects was  $3.4 \pm 0.5$ ; this is not significantly different from the values found before RT, when the ratios were  $3.2 \pm 0.4$  and  $2.9 \pm 0.5$ , respectively, for mediastinal and pelvic patients. During the last week of therapy, the T:B cell

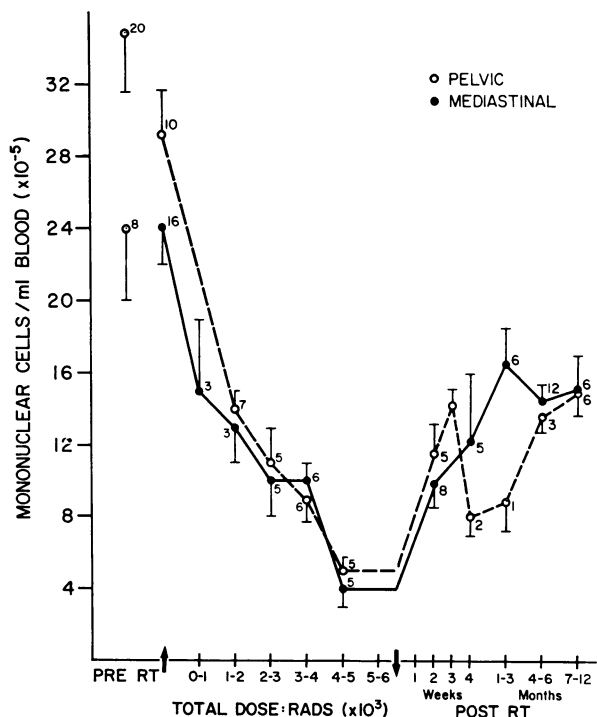


FIGURE 2 The effect of RT on the total numbers of mononuclear cells in the peripheral blood. Symbols are as in Fig. 1.

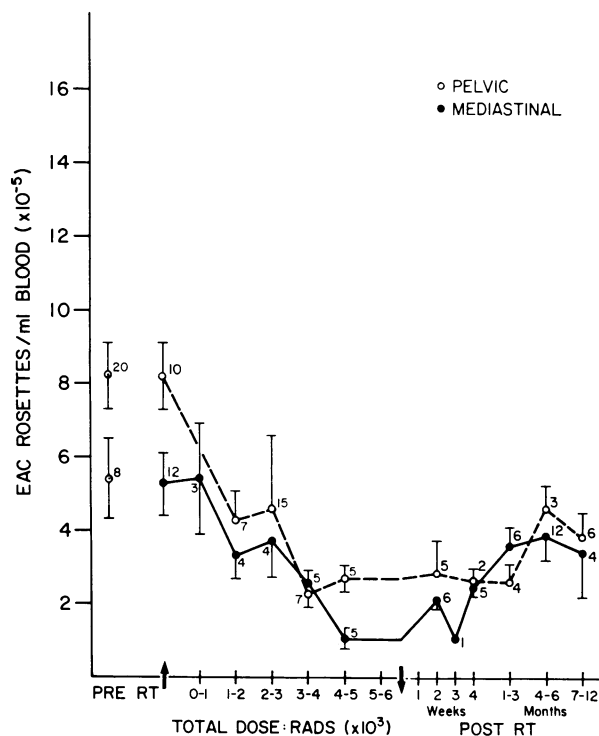


FIGURE 3 The effect of RT on the numbers of EAC rosettes.  $\circ$ --- $\circ$ , 10 patients in group D (pelvic RT);  $\bullet$ — $\bullet$ , 16 patients in group B (mediastinal RT);  $\odot$ , 8 patients receiving mastectomies only (group C), and 20 normal controls (group A) are included. Values are expressed as means of  $n$  determinations; the bar is the SEM.

ratio fell significantly to  $1.9 \pm 0.4$  ( $P < 0.01$ ) for mediastinal and  $1.2 \pm 0.5$  ( $P < 0.01$ ) for pelvic RT patients.

*Acute effects of RT.* The effects of RT on total leukocyte and lymphocyte counts are shown in Figs. 1 and 2. The treatment caused moderate lymphopenia, first evident at 1,000 rads in patients receiving pelvic and 2,000 rads in patients receiving mediastinal RT. Recovery from lymphopenia is quite variable. In some patients, recovery seemed to be complete within 1–3 wk, but then the lymphocyte count dropped again and remained low for months.

A rapid decline is detected in both T and B lymphocytes during RT, when lymphocytes that form spontaneous (E) or complement receptor (EAC) rosettes are enumerated (Fig. 3 and 4). Active E-RFC, prepared by the method of Wybran, underwent essentially the same changes as total E-RFC (Fig. 5). The number of T cells dropped very rapidly and profoundly during RT; a least squares estimate of the slope of the line was  $-2.04 \pm 0.23$  for group B (mediastinal RT) and  $-2.74 \pm 0.42$  for group D (pelvic RT). EAC-RFC decreased more slowly ( $P < 0.01$ ); the slope was  $-0.85$  for both groups B and D. Cells bearing SIg dropped 50% after 2,500–5,000 rads (Table VI).

TABLE VI  
Number of Lymphocytes Bearing SIg in the Blood of  
Normal Subjects and Patients Receiving RT

	SIg	P†
Normal subjects	7.2±0.5 (35)*	>0.5
Pre RT	6.5±1.1 (7)	—
1,800–2,500 rads	3.4±1.1 (6)	<0.05
2,500–5,000 rads	2.6±0.8 (4)	<0.02
After RT (1–8 wk)	2.9±0.9 (6)	<0.05

\* Number of positive lymphocytes  $\times 10^5$ /ml of peripheral blood, mean  $\pm$  SEM (number of subjects).

† Student's *t* test, compared versus number of positive cells before RT.

*In vitro* responses to mitogen stimulation. Many lymphocytes are lost during RT; in addition, those that remain in the circulation lose their ability to incorporate [<sup>3</sup>H]TdR in response to mitogenic stimulation (Figs. 6 and 7). These tests were performed with constant numbers of mononuclear cells, so the decline in functional capacity, to the extent that this is reflected in the response to mitogens, is in addition to the decline in numbers.

During the course of RT, the [<sup>3</sup>H]TdR incorporation for "nonstimulated" cultures increases slightly. The response to PHA-P, as measured by [<sup>3</sup>H]TdR incorpora-

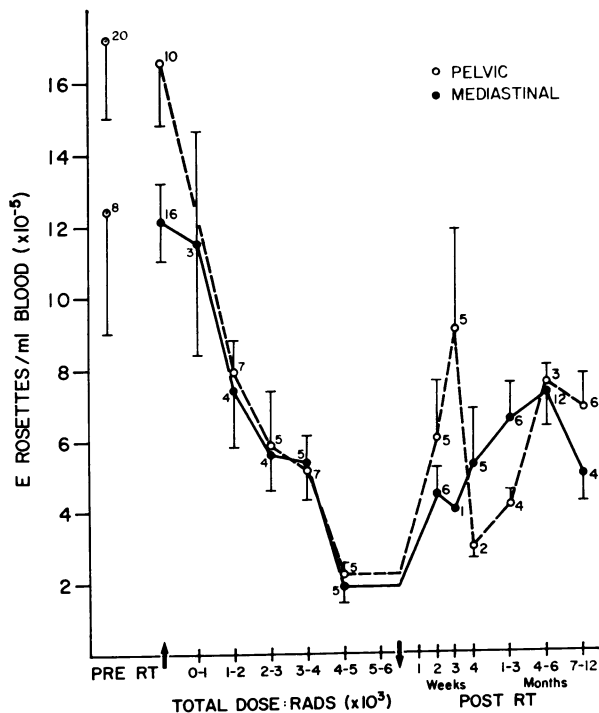


FIGURE 4 The effect of RT on the numbers of total E rosettes. Symbols are as in Fig. 3.

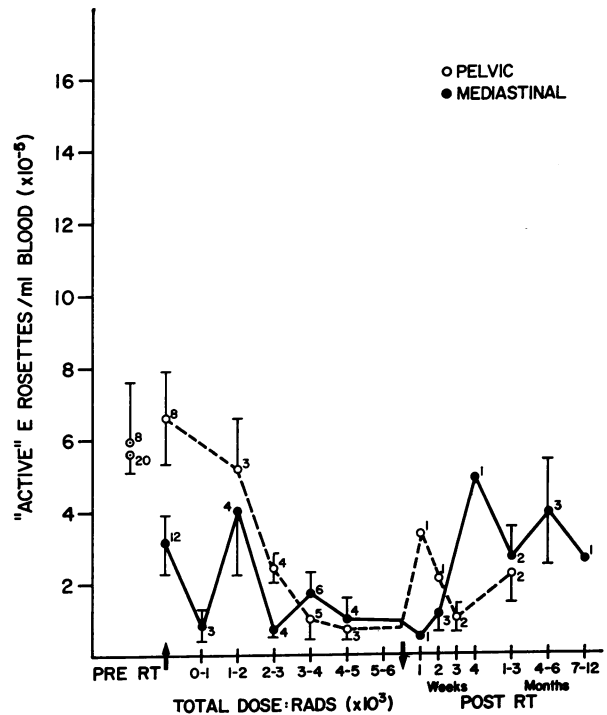


FIGURE 5 The effect of RT on the numbers of E rosettes prepared by Wybran's method for active E rosettes. Symbols are as in Fig. 3.

tion, declines by the time 2,000 rads have been delivered to the mediastinum ( $P < 0.02$ ) or 3,000 rads to the pelvic area ( $P < 0.01$ ). More data are needed to determine if these differences in rapidity of decline are significant. At the end of treatment (approximately 5,000 rads), the response of both groups is profoundly depressed. The response to PWM is also reduced by RT, beginning at 2,000–3,000 rads.

*Recovery of lymphocyte subpopulations after radiotherapy.* Substantial recovery of total mononuclear cells and RFC is evident within 2 wk. The first phase of rapid recovery (about 3 wk) is followed by a second phase of very slow increase (Figs. 3–5). In some patients, especially pelvic RT patients, T cell numbers show a second decline at about 1 mo. The present data show no significant recovery of SIg-bearing cells, but this point requires further investigation (Table VI). Recovery of functional capacity is slower than recovery of either cell number or RFC. The response to PHA-P shows a biphasic recovery curve; modest recovery is seen at 2 wk, followed by a sharp drop at 4 wk ( $P < 0.001$  pelvic,  $P < 0.05$  mediastinal) and a subsequent recovery at 1–3 mo ( $P < 0.05$  pelvic,  $P < 0.1$  mediastinal). 1 yr later the lymphocyte response to PHA-P is still less than 50% of the initial response. Thymidine incorporation after PWM stimulation shows a similar recovery curve.

## DISCUSSION

This report describes a prospective study of lymphocyte subclasses in patients undergoing prophylactic RT for cancer. It deals with the acute changes during RT in the numbers and proliferative capacity of lymphocytes and the relationship of thymic radiation to these changes. We have found that both T and B cells undergo rapid depletion during RT, whether or not the thymus is included in the treatment field. T cells disappear more rapidly and their eventual drop is somewhat greater than that of B cells.

*Patients.* Patients from the NSABP study were chosen as ideal subjects for this research because of the careful documentation and standardized treatment of these patients and because randomized controls not receiving RT are available. NSABP patients receive radiation to part but not all of the thymus. Two patients receiving similar RT for other tumors were included in group B, as the results were similar. More recent studies of two patients receiving total thymic radiation suggest that sparing part of the thymus has no effect on the

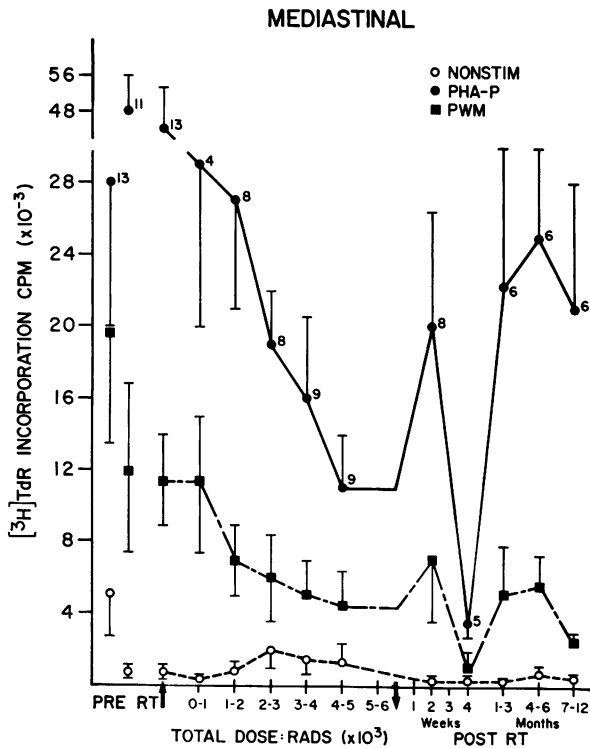


FIGURE 6 The effects of RT on the mitogen-induced DNA synthesis in PBL of 13 breast cancer patients undergoing RT. The values obtained for 13 normal subjects and 11 breast carcinoma patients not receiving RT are illustrated to the left of the figure. ●—●, PHA-P; ■---■, PWM; ○---○, unstimulated control. Values are means of *n* determinations  $\pm$ SEM. Not all patients were tested at each time period.

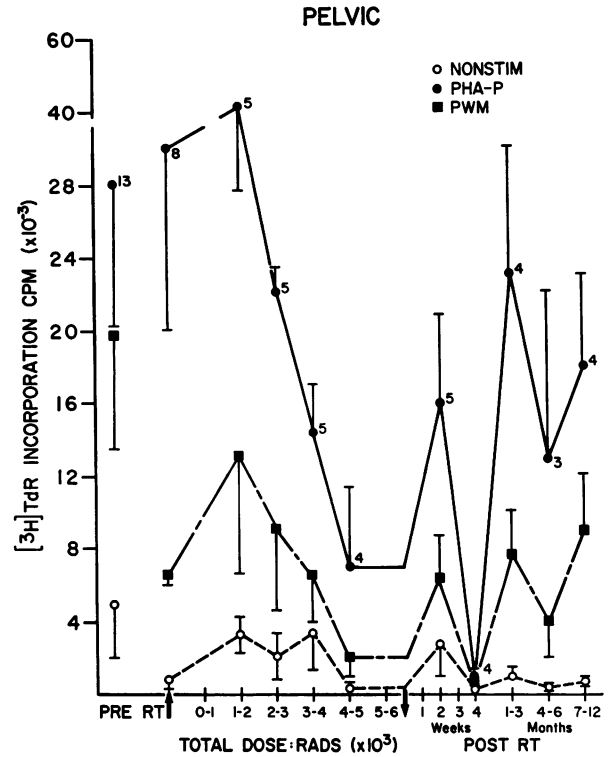


FIGURE 7 Values for 8 patients receiving pelvic RT, along with the values obtained for 13 normal subjects. Symbols are as in Fig. 6.

acute changes in lymphocyte populations (Byfield, P. E., J. A. Stratton, and J. E. Byfield. Unpublished data.). Patients receiving pelvic RT were chosen as controls for the effect of irradiation of tissues (including the lymphoid tissues—blood, lymphatics, bone marrow, and a portion of the gut-associated lymphoid tissues). The latter patients receive a similar dose and rate of RT, with the exception of cervical cancer patients, who also receive radium implants. Data presently available do not support a substantive difference between patients receiving external beam therapy only and those who also have radium implants. Breast cancer patients had all undergone surgery recently (2–5 wk before RT), while the pelvic RT patients had not received surgery before the study. This may be responsible for the depressed T cell levels found in group B and before RT, though others have not found such an association (25).

*Role of thymic radiation in acute lymphopenia.* One purpose of this study was to investigate the hypothesis that a radiation-induced thymic defect was responsible for changes in lymphocyte numbers and subpopulations. For this purpose, patients who had received RT to the mediastinal and pelvic areas, respectively, were compared. Both groups showed a pronounced lymphopenia after receiving about 3,000 rads (Fig. 2 and ref. 24).



Thoracic radiation of mice (2,000 rads in 5 days) caused a similar rapid but fully reversible lymphopenia (26).

Tissues containing lymphoid cells in the treatment fields, in addition to the thymus, are blood, lymphatics, and bone marrow. It has been shown that extracorporeal irradiation of blood *alone* can cause profound lymphopenia (27, 28). Pelvic RT may also expose gut-associated lymphoid tissue to radiation damage. It should also be noted that RT causes nonspecific stress, which may in itself be immunosuppressive (29, 30).

Studies in mice have shown that irradiation of the thymus alone (one dose of 150 rads) causes no lymphopenia in mice, although it alters the proportion of small to large lymphocytes in the blood (31). If some bone marrow is shielded during whole body irradiation, the irradiated thymus is rapidly repopulated by precursors from the bone marrow (31, 32), unless a dose of at least 3,000 rads at one time has been delivered to the organ (33). Thus the thymocytes killed by radiation in these patients are probably replenished rapidly. Circulating T cells, however, seem to be depleted for a longer time.

Comparison of the patients receiving pelvic and mediastinal RT indicates that the alterations in lymphocyte numbers are similar in the two groups. This does not support the inference from the work of Stjernsward, Jondal, Vanky, Wigzell, and Sealy (5) that irradiation of the thymus per se is the primary cause of the alteration in circulating lymphocyte populations. It is clear that thymic radiation is not responsible for acute lymphopenia.

*Relative radiosensitivity of various lymphocyte subpopulations.* When subpopulations of circulating lymphocytes are enumerated during RT, T cells appear to be more radiosensitive than B cells. A decrease in both T and B cells can first be detected between 1,000 and 2,000 rads. When the number of T and B rosettes is plotted against radiation dose (least mean squares method) the slope of the T cell decrease is significantly greater than that of the B cells ( $P < 0.01$ ). The measurement of small numbers of B cells by the EAC rosette technique is subject to question because of possible confusion with monocytes, which also bear complement receptors (11), but the conclusion is borne out by studying a second B cell marker, SIg. The ratio of T to B cells fell significantly in patients receiving both pelvic and mediastinal RT; the overall loss during RT was 85% of E rosettes, but only 75% of EAC rosettes, and 60% of cells bearing SIg. (See also Results: Isolation of lymphocyte subpopulations). Such a decrease in ratio was originally shown by Stjernsward et al. (5).

The ability of lymphocytes to synthesize DNA on mitogen stimulation is quite radiosensitive. Although methods have varied and results differed, most workers agree that RT causes a decrease in the response to

mitogens (5, 34-38). A few have found that RT does not effect the lymphocyte response to mitogens (37, 39, 40). The time after cessation of RT at which the mitogen stimulation is measured is crucial (see Fig. 6-7).

Since both T and B cells show substantial partial recovery within 2-3 wk after the end of therapy (34; and our unpublished data), studies of chronic post-therapy depression cannot be compared directly with studies of acute depression. In general, workers who have found little or no decrease in function have measured the response at 72 h by morphological transformation (37, 39) or thymidine incorporation (39), while those finding depression have measured thymidine incorporation at later times. It may be that there exist, in the blood of patients after RT, radiation-damaged cells that can form rosettes, transform to blasts, and incorporate thymidine, but that die after a few days. It has been shown that morphological transformation may not correlate with [ $^3\text{H}$ ]TdR incorporation (39).

The decrease in ability to synthesize DNA in response to mitogen stimulation is much greater than the decrease in number of lymphocytes during RT. The response of a constant number of isolated lymphocytes to stimulation with PHA or PWM is markedly decreased by 3,000-5,000 rads (Fig. 7-8). Using whole blood cultures in which the number of lymphocytes was not held constant, Jenkins, Olson, and Ellis (36), who studied lung cancer patients during and after RT, also found that the DNA synthetic capacity decreased more than the lymphocyte count.

It appears that the acute decrease in numbers of T and B lymphocytes induced by local RT and the decreased capacity of the remaining blood lymphocytes to divide in response to mitogens are caused by irradiation of lymphocytes in the recirculating pool, rather than by thymic irradiation. On the basis of these studies of acute radiation-induced lymphopenia, we can tentatively conclude that thymic shielding without changing the overall dose of radiation is unlikely to modify the acute lymphopenia induced by mediastinal irradiation.

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