

# Long Term Effects of Radiation on T and B Lymphocytes in Peripheral Blood of Patients with Hodgkin's Disease

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**ABSTRACT** Total lymphocyte counts, and the percentage of T and B lymphocytes and monocytes in untreated patients with Hodgkin's disease were not significantly different from those observed in normal donors. At the completion of radiotherapy, the mean total lymphocyte count of 503/mm<sup>3</sup> was 4 SD below the mean for normal controls. Although a group of 26 patients in continuous complete remission from 12 to 111 mo after radiation treatment regained normal total numbers of lymphocytes and monocytes, they exhibited a striking T lymphocytopenia and B lymphocytosis. Concomitantly, there was a significant increase of null (neither T nor B) lymphocytes.

The response of peripheral blood lymphocytes to phytohemagglutinin, concanavalin A, and tetanus toxoid before treatment was significantly impaired. 1-10 yr after completion of treatment there seemed to be little or no recovery of these responses. The capacity of peripheral blood lymphocytes to respond to allo-antigens on foreign lymphocytes in vitro (mixed lymphocyte reaction) was normal in nine untreated patients. However, the mixed lymphocyte reaction was markedly impaired during the first 2 yr after treatment. There was a partial and progressive restoration of the mixed lymphocyte reaction during the next 3 yr, and normal responses were observed in patients in continuous complete remission for 5 yr or more. The in vivo response to dinitrochlorobenzene was also examined. 88% (15/17) of patients initially sensitive to dinitrochlorobenzene were anergic to the allergen at the completion of a course of radiotherapy, but nine of these regained their hypersensitivity response during the 1st yr after treatment.

This data suggests that there is a sustained alteration in both the number and function of circulating T cells after radiation therapy in patients with Hodgkin's disease which may persist for as long as 10 yr after treatment. The restoration of cell mediated immune functions after radiotherapy is time dependent and its kinetics may differ for various T-cell functions. The implications of these findings with respect to the state of immunological competence after radiotherapy are discussed.

## INTRODUCTION

Radiation induced alterations in the number and function of peripheral blood lymphocytes have recently been described in patients treated with radiotherapy for carcinoma of the breast (1-6), lung (7-9), bladder (7-10), uterine cervix (2, 6, 11), testicular seminoma and carcinoma (12, 13), Hodgkin's disease, (14-19) and in children receiving prophylactic craniospinal irradiation for acute lymphoblastic leukemia (13, 20). Most studies have demonstrated an acute lymphocytopenia and suppression of immune function, shortly after the initiation of treatment. Several investigators have shown that a partial recovery may occur within the first 18 mo after the completion of treatment (6, 8, 10-14). There are, however, few data available on the long term effects of radiation therapy on the number and immune function of peripheral blood lymphocytes in patients in continuous remission many years after treatment.

In the present study, we describe long term changes in the number, cell surface characteristics, and in vitro function of peripheral blood lymphocytes in a group of patients in continuous complete remission after an intensive course of radiation therapy for Hodgkin's disease.

## METHODS

*Patient selection and staging.* The study comprised a group of 227 patients with Hodgkin's disease referred to

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Stanford University Medical Center between 1965 and 1975. The histologic diagnosis of the initial biopsy specimens was confirmed and classified according to the Rye modification (21) of the Lukes and Butler classification (22). The patients were thoroughly evaluated for extent of disease before therapy as has been detailed elsewhere (23, 24) and staged according to the scheme proposed at the Ann Arbor Conference (25).

The immunological studies were performed in two groups of patients. The first consisted of 148 untreated patients, in whom studies were performed after initial biopsy but before any staging procedures. All but a few of these patients (those with stage IV disease) were eventually staged with bipedal lymphangiography and laparotomy with splenectomy. 8 patients had pathological stage IA, 2, stage IB, 54, stage IIA, 16, stage IIB, 32, stage IIIA, 15, stage IIIB, 14, stage IVA, and, 8, stage IVB disease.

The second group consisted of 79 treated patients (18 patients had stage IA, 1, IB, 33, IIA, 6, IIB, 14, IIIA, 5, IIIB, 1, IVA, and 1, IVB). At the time of the immunological studies, all tested patients were in continuous complete remission from 1-10 yr after the initial course of treatment. All patients were treated initially with intensive radiotherapy delivered by a 6 MeV linear accelerator employing techniques of local, extended field, or total lymphoid irradiation as previously described (24). 10 of the patients also received prophylactic multiple drug (MOPP)<sup>1</sup> chemotherapy after irradiation (26).

In addition, 86 normal persons, consisting of laboratory personnel, physicians, and nurses, roughly similar in age and sex distribution to the patients with Hodgkin's disease, were tested.

**Lymphocyte separation.** Peripheral blood was collected in heparin and lymphocytes were separated on a Ficoll-Hypaque gradient as previously described (27).

**Identification and enumeration of T and B lymphocytes and monocytes.** T lymphocytes were identified by a complement-dependent antibody cytotoxicity assay according to Bobrove et al. (28). This method utilizes an anti-T-cell serum developed by immunizing an adult goat with viable human thymus cells, and subsequent absorption of the crude antiserum with malignant B cells. The percentage of cells killed by the antiserum was determined by trypan blue exclusion. T lymphocytes were also identified on the same blood samples by their ability to form spontaneous rosettes with sheep erythrocytes (E rosettes), by using the method of Bentwich et al. (29). At least 200 cells were counted, and each test was done in triplicate. Only lymphocytes with at least three erythrocytes attached were considered E rosettes.

B lymphocytes were identified by staining the Ig-bearing cells with a fluorescein-conjugated polyvalent rabbit anti-human-Ig-antiserum (28). Monocyte contamination was determined by staining with alpha-naphthol acetate (Sigma Chemical Co., St. Louis, Mo.) according to Yam et al. (30). The percent monocyte contamination and the cytotoxic index obtained with the anti-T-cell serum were used to calculate

the percentage of T cells in the peripheral blood as follows:

% T cells = cytotoxic index (%)

$$\left( \frac{100}{100 - \% \text{ monocyte contamination}} \right)$$

The percentage of B cells was independent of the monocyte contamination, since only small cells were examined for surface Ig staining, thereby excluding monocytes (28).

These procedures were performed immediately after Ficoll-Hypaque purification of freshly drawn blood. However, in some cases the same tests were repeated after incubation of the cell suspensions in RPMI-1640 and 20% fetal calf serum for 18-24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The survival of the cells after incubation, determined by cell counts, was greater than 90%. The percentage of viable cells as determined by trypan blue exclusion was greater than 98%.

The absolute numbers of T and B lymphocytes in the peripheral blood were calculated by multiplying the percentage of T and B lymphocytes by the absolute lymphocyte count computed from the total leukocyte and differential counts obtained on the same day (31).

**Mixed lymphocyte reactions.** The ability of lymphocytes to respond to alloantigens in vitro was tested by a one-way mixed lymphocyte culture microassay according to Sasazuki et al. (32). In brief, 50,000 Ficoll-Hypaque purified peripheral blood lymphocytes from normal individuals or patients were mixed with 50,000 Ficoll-hypaque purified stimulator cells from unrelated normal donors or patients with Hodgkin's disease. Stimulator cells were inactivated by radiation, receiving a single dose of 6,000 rads from a radioactive cesium<sup>137</sup> source (Mark I model 25 Irradiator, J. L. Shepherd and Associates, Glendale, Calif.) in air at room temperature. Cells were then mixed in 0.2 ml of RPMI-1640 medium with 25 mM Hepes buffer supplemented with L-glutamine, streptomycin, penicillin, and 10% heat inactivated human AB serum, in round bottom microtiter trays (Linbro Chemical Co., Inc., New Haven, Conn.). The mixtures were cultured for 6 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, and subsequently pulsed with 1 µCi [<sup>3</sup>H]thymidine (2 Ci/mmol, Schwarz-Mann Radiochemicals, Rockville, Md.) for 16 h. The rate of DNA synthesis was estimated by measurement of the incorporation of the radioactive thymidine into the responder cells.

**Tetanus toxoid induced transformation of lymphocytes.** The lymphocyte response to a specific antigen was investigated by mixing 10 µg of tetanus toxoid with Ficoll-Hypaque purified peripheral blood lymphocytes in 0.2 ml of RPMI-1640 medium with 25 mM Hepes buffer supplemented with L-glutamine, streptomycin, penicillin, and 10% heat inactivated human AB serum in flat bottom microtiter trays. The cells were cultured for 6 days and pulsed with [<sup>3</sup>H]thymidine for 16 h as described above. Except where otherwise stated, 200,000 cells were used per culture.

**Stimulation of lymphocytes by lectins.** Blastogenic stimulation of peripheral blood lymphocytes by phytohemagglutinin (PHA, Wellcome Reagents Ltd., Beckenham, England) and concanavalin A (Con A, crystallized X3 and lyophilized, Miles Laboratories, Inc., Kanakee, Ill.) was tested according to the method of Levy and Kaplan (33). This microassay measures the degree of stimulation of protein synthesis by lectins (PHA, Con A) by comparing the increase of [<sup>3</sup>H]leucine incorporation obtained at various concentrations of the mitogen with the amount of incorporation by unstimulated cells. The degree of stimulation of protein synthesis (stimulation ratio) was expressed in terms

<sup>1</sup> **Abbreviations used in this paper:** Con A, concanavalin A; DNCB, dinitrochlorobenzene; Ig, immunoglobulin; MLR, mixed lymphocyte reaction (culture); MOPP, multiple drug chemotherapy with nitrogen mustard, oncovin, prednisone, and procarbazine; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; TLI, total lymphoid irradiation.

of the ratio of counts incorporated in the presence of the mitogen to the counts incorporated simultaneously in the saline control.

**Delayed hypersensitivity to DNCB.** Patients were tested for their ability to develop delayed hypersensitivity skin reactions to 2,4-dinitrochlorobenzene (DNCB) before staging laparotomy or therapy. In the majority of cases, sensitization was performed with 500  $\mu$ g DNCB as previously described (34); an early subgroup of 34 patients was sensitized with 2,000  $\mu$ g DNCB. Challenge was performed 10–14 days later with 100  $\mu$ g of the chemical applied to the skin of the opposite forearm. A reaction was considered positive if both erythema and induration developed with or without vesiculation 24–96 h after challenge. In 66 patients, repeated challenges were also performed after completion of treatment at 3–6 mo intervals for 1–10 yr, until a positive response was observed.

## RESULTS

**Absolute lymphocyte counts.** Table I summarizes the mean lymphocyte counts of 22 normal donors, 61 patients with untreated Hodgkin's disease, and 26 patients with treated Hodgkin's disease in continuous long term complete remission. The mean total lymphocyte count in the group of treated patients was not significantly different from the mean counts of normal donors or of patients with untreated Hodgkin's disease. All patients in the treated group had received radiation therapy 12–111 mo before testing, and three patients had also received six courses of MOPP chemotherapy (Table II).

Absolute lymphocytopenia, defined as values more than 2 SD below the mean for normal donors, (mean  $\pm$

SD =  $2,038 \pm 295$  lymphocytes/mm<sup>3</sup>) was observed in the present study in 24/61 (39%) of the patients with untreated Hodgkin's disease. At the completion of radiotherapy, all patients manifested severe lymphocytopenia (Table II). The mean count for this group was 503 lymphocytes/mm<sup>3</sup> and all patients had values more than 4 SD below the mean for normal donors. By 12–111 mo after completion of radiation treatment, only 7/26 (27%) had absolute lymphocytopenia. There was, however, no correlation between pretreatment lymphocytopenia and that observed 12–111 mo later. Only three patients in whom lymphocytopenia was observed before treatment also had absolute lymphocytopenia 21, 55, and 60 mo after completion of treatment. The other four patients with posttreatment lymphocytopenia had normal counts before treatment; conversely, eight other patients with pretreatment lymphocytopenia had normal lymphocyte values 12–111 mo after radiation. There was also no correlation between either the treatment modality employed or the duration of continuous complete remission and the presence of absolute lymphocytopenia (Table II).

**B lymphocyte counts.** Tables I and II show a summary of the results of quantitation of B cells identified as surface Ig-bearing cells. The mean percentage of Ig-bearing cells in normal donors was 20% of the total lymphocytes (Table I), and the mean absolute B lymphocyte count  $\pm$  SD was  $407 \pm 101$ . Although the mean percentage of Ig-bearing cells in patients with untreated

TABLE I  
*T and B Lymphocytes in Normal Donors and Patients with Hodgkin's Disease*

	Cells from Ficoll-Hypaque gradients				Peripheral blood cells			
	Percent monocytes	Cytotoxic index	E rosettes	Lymphocytes		Total lymphocytes	T-lymphocytes	B-lymphocytes
		%	%	% B	% T <sup>‡</sup>	per mm <sup>3</sup> *	per mm <sup>3</sup> * <sup>§</sup>	per mm <sup>3</sup> *
Normal donors (22 patients)	17 $\pm$ 1.2 <sup>‡</sup> (10–30)	64 $\pm$ 1.6 (49–80)	64.8 $\pm$ 0.6 (47–83)	20 $\pm$ 1.2 (13–30)	77 $\pm$ 1.5 (65–91)	2,038 $\pm$ 73 (1,400–2,700)	1,600 $\pm$ 76 (1,092–2,400)	407 $\pm$ 25 (268–640)
Untreated Hodgkin's disease stage I–II (35 patients)	18 $\pm$ 1.4 (3–38)	64 $\pm$ 1.6 (43–86)	51.5 $\pm$ 3.4 (29–82)	19 $\pm$ 1.3 (7–34)	77 $\pm$ 1.2 (61–90)	1,634 $\pm$ 132 (703–3,720)	1,271 $\pm$ 98 (492–2,567)	327 $\pm$ 37 (77–680)
Untreated Hodgkin's disease stage III–IV (26 patients)	27 $\pm$ 3.7 (5–79)	61 $\pm$ 3.5 (10–80)	52.4 $\pm$ 3.9 (40–71)	17 $\pm$ 1.5 (7–33)	83 $\pm$ 2.3 (48–98)	1,647 $\pm$ 200 (200–5,256)	1,355 $\pm$ 170 (261–4,362)	285 $\pm$ 40 (44–841)
Hodgkin's disease treated and no evidence disease (26 patients)	20 $\pm$ 3.1 (0–72)	32 $\pm$ 2.3 (10–53)	38.1 $\pm$ 1.6 (12–55)	37 $\pm$ 2.2 (20–60)	41 $\pm$ 2.8 (15–74)	1,985 $\pm$ 152 (754–3,815)	793 $\pm$ 66 (287–1,484)	725 $\pm$ 66 (150–1,526)

\* Calculated values; those for total lymphocytes are derived by multiplying the total leukocyte count by the percentage of lymphocytes in the differential count; those for T and B lymphocytes by multiplying the total lymphocyte count by the measured percentages of T and B lymphocytes, respectively. Data on these normal donors and on 42 of the untreated patients (20 Stage II, 22 Stages III and IV) have been previously published (31).

<sup>‡</sup> Mean  $\pm$  SE (Range).

<sup>§</sup> Calculated from cytotoxic index.

**TABLE II**  
*T and B Lymphocytes and Immune Functions in Patients with Hodgkin's Disease, Previously Treated  
and in Continuous Complete Remission*

Type	Initial stage	Total lymphocytes per mm <sup>3</sup> before XRT	Initial treatment	Total lymphocytes per mm <sup>3</sup> at XRT completion	Months after XRT	Cell from Ficoll-Hypaque gradients					Peripheral blood cells		
						E-rosettes	Cyto-toxic index	Mono-cytes	Lymphocytes		Total	B-cells	T-cells†
						%	%	%	%	%		per mm <sup>3</sup>	per mm <sup>3</sup>
NSHD	IA	1,239	TLI*	686	55	42	39	10	30	43	1,254	376	539
NSHD	IA	1,946	TLI	216	61	28	18	26	32	24	1,292	413	310
LPHD	IA	3,204	Mantle	ND	109	47	26	32	20	38	754	150	286
NSHD	IIB	1,274	TLI	400	12	12	22	25	33	29	1,491	492	432
NSHD	IIA	1,292	TLI	697	13	37	49	2	24	74	1,938	465	1,434
NSHD	IIB	1,612	TLI	572	14	33	26	11	38	29	1,540	585	446
NSHD	IIA	1,391	TLI	270	21	37	26	37	51	41	1,276	650	523
NSHD	IIIEA	1,495	TLI + MOPP	576	30	42	38	26	59	51	2,470	1,457	1,259
NSHD	IIA	1,500	Mantle	294	33	44	53	8	33	58	1,452	479	842
NSHD	IIA	2,000	TLI	216	49	35	12	18	41	15	2,712	1,112	406
NSHD	IIA	4,066	TLI	845	54	38	21	40	49	35	2,436	1,195	852
NSHD	IIA	972	TLI	498	55	33	38	7	22	41	2,883	635	1,182
NSHD	IIIEB	2,040	TLI	768	56	46	44	36	24	69	1,280	307	883
NSHD	IIIEA	944	TLI	780	60	39	50	6	44	52	1,521	669	790
MCHD	IIA	1,914	UP. ABD.	435	62	35	20	33	29	30	2,470	713	741
NSHD	IIA	1,560	TLI	ND	83	38	17	2	44	18	2,350	1,034	1,128
HD	IIB	815	TLI	ND	88	48	43	25	29	57	2,604	756	1,484
NSHD	IIIEA	2,325	TLI	468	104	43	32	16	38	38	2,701	1,026	1,026
HD	IIA	2,106	TLI	ND	111	45	24	18	25	45	2,166	541	974
MCHD	IIISB	864	TLI + <sup>198</sup> Au	252	20	48	47	14	37	55	1,452	537	798
NSHD	IIIESB	960	TLI + MOPP	280	22	27	27	10	30	30	2,160	648	648
MCHD	IIISA	2,940	TLI + <sup>198</sup> Au	980	36	41	34	0	29	34	3,564	1,033	1,211
NSHD	IIIB	1,330	TLI + MOPP	ND	42	30	39	16	42	46	1,525	640	701
NSHD	IIISA	1,710	TLI + <sup>198</sup> Au	640	56	22	10	72	40	26	3,815	1,526	991
NSHD	IIISA	968	TLI + <sup>198</sup> Au	350	60	36	38	8	55	41	1,386	762	568
HD	IVHA	1,920	TLI + <sup>198</sup> Au	108	101	41	31	30	60	44	1,092	655	480

\* Mantle, mantle field irradiation only; <sup>198</sup>Au, intravenous colloidal radioactive gold; HD, Hodgkin's disease; LPHD, lymphocyte predominance HD; MCHD, mixed cellularity HD; NSHD, nodular sclerosing HD; XRT, radiotherapy.

† Calculated from cytotoxicity index.

Hodgkin's disease was not significantly different from the percentage in normal donors, the mean absolute B lymphocyte count was significantly lower ( $P < 0.05$ ) than that of normal donors (Table I). Of the 61 patients with untreated Hodgkin's disease 23 (38%) had absolute B lymphocytopenia, defined as counts more than 2 SD below the mean for normal donors. Immediately after completion of radiotherapy, there was a severe depletion of peripheral blood B lymphocytes in five patients tested (mean  $\pm$  SD =  $47.2 \pm 31.3$  B lymphocytes/mm<sup>3</sup>). At 12–111 mo after radiotherapy, both the mean percentage and the mean absolute number of B lymphocytes were significantly higher ( $P < 0.01$ ) than those observed in normal donors. Of the 26 treated patients, 16 (61%) had an absolute B lymphocytosis and only one had an absolute B lymphocytopenia (Table II). The degree of B lymphocytosis was, however, mild and the highest count observed was 1,526 B lymphocytes/mm<sup>3</sup>.

**T lymphocyte counts.** The mean T lymphocyte count  $\pm$  SD in 22 normal donors as detected by the cytotoxicity

assay was  $1,600 \pm 303$ . There was no significant difference between the mean of the untreated patients and that of the normal controls (Table I), although 20/61 (33%) of the untreated patients had an absolute T lymphocytopenia. Nearly all patients with T lymphocytopenia had an associated B lymphocytopenia and absolute total lymphocytopenia. There was a striking T lymphocytopenia in five patients tested immediately after radiotherapy (mean  $\pm$  SD =  $150.2 \pm 134.2$ ) and also in the group of 26 patients tested 12–111 mo after completion of treatment (Table I, II). The mean T lymphocyte count of 793 lymphocytes/mm<sup>3</sup> (Table I) for the latter group was significantly lower than the mean value for normal donors ( $P < 0.01$ ). Absolute T lymphocytopenia occurred in 19/26 (73%) of the patients in this group.

In the treated patients, there was no correlation between the presence of T lymphocytopenia and either the treatment technique employed or the time interval from completion of treatment (Table II). Even patients

treated more than 8 yr previously had absolute T lymphocytopenia.

**Incidence of "null" lymphocytes.** "Null" cells are defined as cells, identified morphologically as small lymphocytes, which do not carry surface markers of either B lymphocytes or T lymphocytes. Examination of the frequency distributions of "null" lymphocytes shows that more than 90% of the normal donors and patients with untreated Hodgkin's disease had  $\leq 14\%$  "null" lymphocytes, whereas 59% of the treated patients in long term remission had 15–49% "null" lymphocytes. There was no correlation between the presence of an increased percentage of "null" lymphocytes and the interval from completion of radiotherapy (Table II).

**Percentage of monocytes.** Table I shows a summary of the percentages of monocytes contaminating the Ficoll-Hypaque gradients. There were no statistically significant differences among the percentages of monocytes in gradient-separated cells from the peripheral blood of normal donors, patients with untreated Hodgkin's disease, or patients in long term remission after radiotherapy.

**T lymphocytes by E rosette method.** Confirming our earlier observations in a smaller number of patients (31), the mean percentage of T lymphocytes identified by the E rosette test in untreated patients (52.3%) was significantly lower ( $P < 0.05$ ) than the percentage detected by the cytotoxicity assay (62.4%) (Table III). In contrast, the mean percentage of T cells detected by the E rosette method in the group of patients in long term continuous remission after radiotherapy was significantly higher ( $P < 0.05$ ) than the percentage determined by the cytotoxicity assay (Tables I, II, III). The percentage of T lymphocytes in the treated patients was

significantly lower by both methods than the corresponding percentages for normal donors and untreated patients.

**Effect of overnight incubation on T cell determination.** Incubation of peripheral blood lymphocytes from patients with untreated Hodgkin's disease in medium containing 20% fetal calf serum is followed by restoration of the percentage of E rosette forming cells up to the levels of T lymphocytes detected by the cytotoxic antibody assay (Table III). Incubation of Ficoll-Hypaque purified peripheral blood lymphocytes from treated patients for 24 h in 20% fetal calf serum did not change the percentage of E rosette forming cells, but significantly increased the percentage of T lymphocytes detected by the cytotoxicity assay from  $30.6 \pm 5.5$  to  $42.6 \pm 4.3\%$  (Table III). Even after incubation in fetal calf serum, the percentage of T lymphocytes by either method remained significantly lower ( $P < 0.01$ ) than the corresponding values observed in normal donors and in patients with untreated Hodgkin's disease.

**Effect of overnight incubation on Ig-bearing cells.** It has recently been shown that fluoresceinated whole rabbit anti-human-Ig-antiserum used to detect surface immunoglobulins on lymphocytes stains both cells producing immunoglobulins endogenously as well as cells binding immunoglobulin via the Fc receptor (35, 36). Staining of the latter cells can be reduced after overnight incubation in 20% fetal calf serum.

Incubation of Ficoll-purified lymphocytes from 12 normal donors for 24 h in RPMI-1640 plus 20% fetal calf serum resulted in a decrease of the mean percentage of Ig-bearing cells from 18.2 to 11.7 (Table III). Similar incubation of Ficoll-purified lymphocytes from 12 treated patients resulted in a reduction of the mean per-

TABLE III  
Percentage of T Lymphocytes by Spontaneous E-Rosette and Cytotoxicity Assays and Percentage of Ig Bearing Lymphocytes (B-cells) in Ficoll-Hypaque Purified Peripheral Blood Lymphocytes of Normal Donors and of Patients with Hodgkin's Disease Before and After 24 h Incubation in RPMI-1640 with 20% Fetal Calf Serum

Group	E-rosettes*		Cytotoxicity*		Ig bearing cells*	
	Before incubation	After incubation	Before incubation	After incubation	Before incubation	After incubation
Normal donors	$66.1 \pm 0.9$ (56–81) (40 donors)	$65.4 \pm 1.2$ (52–85)	$68.3 \pm 2.8$ (38–82) (22 donors)	$73.8 \pm 1.9$ (54–85)	$18.2 \pm 1.8$ (9–29) (12 donors)	$11.7 \pm 1.7$ (2–28)
Untreated Hodgkin's disease	$52.3 \pm 1.5$ (26–82) (57 patients)	$64.0 \pm 1.3$ (32–83)	$62.4 \pm 2.4$ (43–83) (34 patients)	$66.1 \pm 2.5$ (43–90)	ND	ND
Hodgkin's disease treated and no evidence of disease	$36.2 \pm 1.8$ (12–47) (22 patients)	$36.8 \pm 1.7$ (14–48)	$30.6 \pm 5.5$ (10–65) (22 patients)	$42.6 \pm 4.3$ (19–62)	$42.7 \pm 3.3$ (23–65) (12 patients)	$26.4 \pm 4.0$ (6–55)

\* Mean  $\pm$  SE (Range).

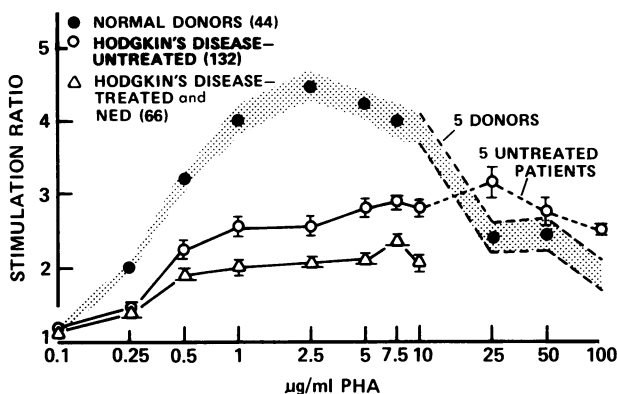


FIGURE 1 PHA stimulation of protein synthesis in peripheral blood lymphocytes. The data are expressed on the basis of the stimulation ratio (ratio of counts per minute in PHA-stimulated cultures to counts per minute in unstimulated controls for each subject at each PHA concentration). The data from 44 normal donors, 132 patients with untreated Hodgkin's disease (all stages), and 66 Hodgkin's disease patients treated with radiotherapy and in complete clinical remission for 1–10 yr after treatments are presented. The data for each group are pooled and presented as the mean  $\pm$  SE of the stimulation ratios at each PHA concentration.

centage of Ig-bearing cells from 42.7 to 26.4. These differences after overnight incubation were both statistically significant ( $P < 0.01$ ).

**Lymphocyte stimulation by PHA.** Fig. 1 summarizes the results of PHA stimulation assays with Ficoll-Hypaque purified lymphocytes obtained from normal donors and from patients with untreated and treated Hodgkin's disease. Optimal stimulation in normal donors occurred at a PHA concentration of 2.5  $\mu$ g/ml (mean stimulation ratio  $\pm$  SE =  $4.45 \pm 0.2$ ) and a sharp decrease was observed at higher or lower concentrations. Confirming an earlier report (33), the present study shows that the mean stimulation ratio in 132 patients with untreated Hodgkin's disease was significantly lower than the normal values at all dose levels between 0.25 and 10  $\mu$ g/ml of PHA (at each dose tested,  $P < 0.01$ ). The decrease in responsiveness to PHA was significantly more pronounced in untreated patients with advanced disease (stages III and IV) than in patients with stages I and II (data not shown). However, even in the latter group, the response was significantly below that of normal donors.

The response to PHA of peripheral blood lymphocytes from 66 radiation-treated patients tested while in long term complete remission is also presented in Fig. 1. The stimulation ratios over a wide range of PHA concentrations were even more profoundly depressed than those for the untreated patients ( $P < 0.01$ ). There was no correlation between the time interval from completion of treatment and the response to PHA. Patients treated as

much as 9–10 yr earlier had a mean stimulation ratio ( $2.39 \pm 0.39$ ) similar to that of patients treated only 1 yr before PHA stimulation ( $2.03 \pm 0.28$ ). There was also no correlation between the extent or topographical localization of the radiation fields and response to PHA. For example, the mean stimulation ratio ( $1.78 \pm 0.20$ ) in patients given only infradiaphragmatic irradiation was as low as that in patients given TLI. No additional suppression was induced by the adjunctive administration of MOPP chemotherapy.

**Lymphocyte stimulation by Con A.** Patients with untreated Hodgkin's disease had a decreased response to Con A (Fig. 2) which was significant ( $P < 0.01$ ) at concentrations of 5 and 10  $\mu$ g/ml. A nearly identical degree of suppression was observed in a group of 19 patients previously treated with radiation and in continuous complete long term remission.

**Lymphocyte stimulation by tetanus toxoid.** Fig. 3 shows the response of peripheral blood lymphocytes from normal donors and patients with Hodgkin's disease to in vitro stimulation by tetanus toxoid (kindly supplied by the Department of Public Health of the Commonwealth of Massachusetts). None of the tested individuals (normal donors or patients with Hodgkin's disease) had received an injection of the toxoid shortly before in vitro testing. It was assumed that all tested individuals had been actively immunized against tetanus sometime in the past, although not all individuals could recall such an event.

As shown in Fig. 3, culture of peripheral blood lymphocytes from normal donors with tetanus toxoid resulted in a significant blastogenic response. Of 40 normal donors tested, 34 (85%) showed activities of the extracted DNA exceeding 20,000 cpm per well (background counts of unstimulated cells did not exceed 1,000

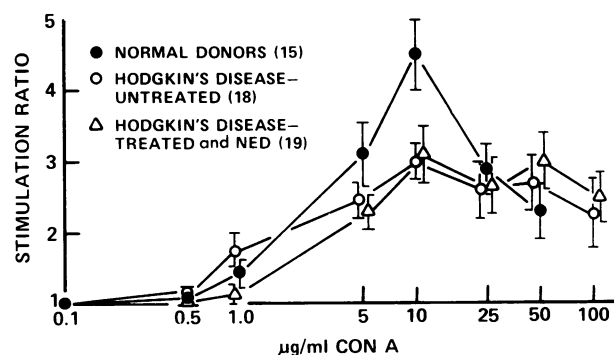


FIGURE 2 Con A stimulation of protein synthesis in peripheral blood lymphocytes from 15 normal donors, 18 patients with untreated Hodgkin's disease, and from 19 patients with Hodgkin's disease treated with radiotherapy and in complete remission for 1–10 yr after treatment. The data for each group are pooled and expressed as the mean  $\pm$  SE of the stimulation ratios at each Con A concentration. (NED, no evidence of disease.)

cpm/well). In contrast, only 3/9 (33%) of the untreated patients ( $P < 0.01$ ) exhibited positive in vitro responses to the tetanus toxoid.

The degree of in vitro stimulation of 200,000 lymphocytes from treated patients was compared with that of 100,000 cells from normal individuals (Fig. 3), since the number of peripheral blood lymphocytes identifiable as T cells is reduced by approximately 50% after therapy. The degree of stimulation of normal individual lymphocytes under these conditions was still significantly higher than that of the treated patients. A further increase in the number of tested lymphocytes from treated patients to 400,000 did not result in a significant increase in the response to tetanus toxoid (Fig. 3) which remained significantly lower than the response of 200,000 cells from normal donors.

**Mixed lymphocyte reaction (MLR).** The capacity of peripheral blood lymphocytes from normal donors and patients with Hodgkin's disease to respond to foreign lymphocytes in vitro is shown in Fig. 4. In the present study, MLR tests resulting in extracted DNA activities greater than 10,000 cpm per well were defined as positive responses, since this was the lower limit of normal responses. It should be noted that only 12/131 (9%) of

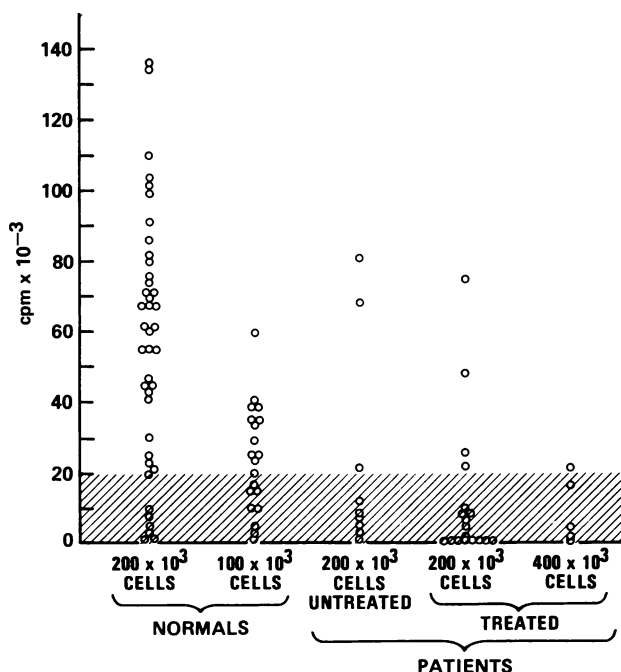


FIGURE 3 Tetanus toxoid stimulated DNA synthesis of peripheral blood lymphocytes from normal donors, patients with untreated Hodgkin's disease, and patients with Hodgkin's disease treated with radiotherapy and in complete remission for 1-10 yr after treatment. The data express the activity of [ $^3\text{H}$ ]thymidine incorporated into the cells per culture. The number of cells indicate the number of cells utilized per culture.

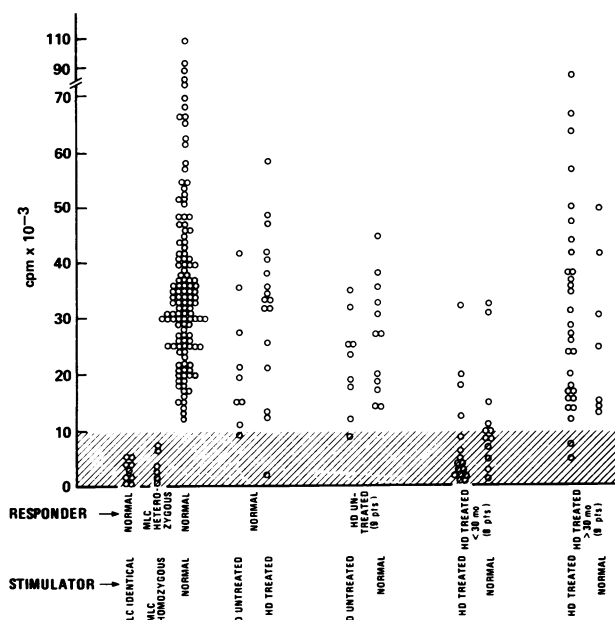


FIGURE 4 MLR of peripheral blood lymphocytes. Various combinations of normal individuals and patients with untreated and treated Hodgkin's disease are presented. The data express the activity of [ $^3\text{H}$ ]thymidine incorporated into the cells per culture (mo, months; pts, patients; HD, Hodgkin's disease).

the normal unrelated combinations showed activities between 10,000 and 20,000 cpm per well. These were arbitrarily defined as weakly positive responses whereas activities higher than 20,000 cpm per well were defined as strongly positive responses.

Cells from nine untreated patients with active Hodgkin's disease (3 patients in stage IA, 2 IIA, 2 IIB, 1 IIIA, and 1 patient in stage IIIB disease) and from 12 patients in long term remission after radiotherapy were found to induce positive responses in 24/26 (92%) random combinations with responder cells from unrelated normal individuals (Fig. 4). It should be noted, however, that 5/9 (55%) of the responses, using untreated patients as donors of stimulator cells, were either subnormal or weakly positive, whereas only 3/17 (18%) of the responses, using stimulator cells from treated patients in complete remission, were weak.

Peripheral blood lymphocytes from patients with untreated Hodgkin's disease were found to respond adequately when stimulated by either normal donor cells or cells obtained from other patients with Hodgkin's disease. In 20/21 (95%) random combinations positive MLR responses were observed (Fig. 4). Recent radiotherapy (which in all cases was TLI) significantly reduced the capacity of patients with Hodgkin's disease to respond to the MLR test. Eight patients tested 12-30 mo after treatment showed adequate responses in only 4/12 (33%) combinations when randomly paired with

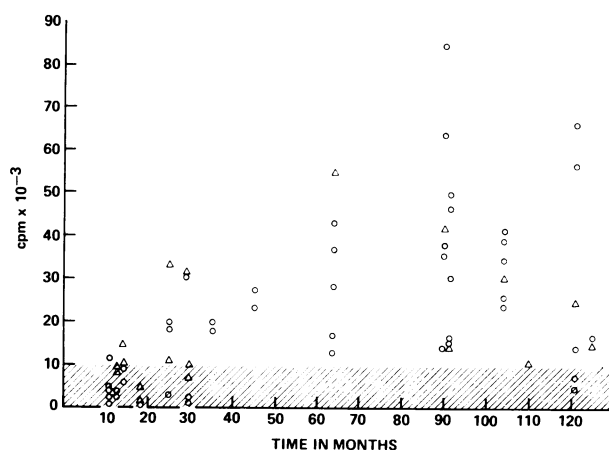


FIGURE 5 MLR of peripheral blood lymphocytes from Hodgkin's disease patients treated with radiotherapy and in complete remission 1-11 yr after therapy; correlation of response with time elapsing from therapy. Stimulator cells were obtained from normal donors ( $\Delta$ ) or other treated patients (O).

normal donor lymphocytes, and in 4/16 (25%) when paired with lymphocytes from other treated patients. In contrast, of 37 combinations in which responder cells were used from patients treated 31 to 120 mo previously, and stimulator cells from unrelated normal donors or other treated patients, 35 (95%) yielded positive responses. Fig. 5 shows the correlation between responses observed in treated patients and the time elapsing from completion of radiotherapy. The MLR was significantly impaired during the first 2 yr after treatment. There was a partial and progressive restoration of the response during the next 2 yr, and normal responses were observed in patient in continuous complete remission for 5 yr or more.

*Delayed hypersensitivity reaction to topical application of DNCB.* A previous report from this institution de-

TABLE IV  
Correlation of Delayed Hypersensitivity Response to DNCB with Absolute Peripheral Blood T-Lymphocyte Counts and PHA Stimulation in Patients with Untreated Hodgkin's Disease

	DNCB (+)	DNCB (-)
T-lymphocytes/mm <sup>3</sup> of peripheral blood (normal = 1,600 $\pm$ 76)*	1,350 $\pm$ 155* (492-2,592) (n = 17)†	1,371 $\pm$ 134 (502-4,362) (n = 33)
Stimulation ratio PHA, 2.5 $\mu$ g/ml (normal = 4.45 $\pm$ 0.20)*	2.85 $\pm$ 0.29 (0.81-6.89) (n = 36)	2.71 $\pm$ 0.15 (0.90-7.18) (n = 76)

\* Mean  $\pm$  SE.

† n = number of individuals tested.

TABLE V  
Delayed Hypersensitivity Response to DNCB before and after Treatment in Patients with Hodgkin's Disease: Patients were Sensitized with 500-2,000  $\mu$ g DNCB before Treatment and Challenged with 100  $\mu$ g 10-14 Days Later and at 3-6 mo Interval after Completion of Treatment

	Before treatment		After treatment	
		%		%
Pos*	17/69	(25)	Pos	12/17 (71)
			Neg	5/17 (29)
Neg†	52/69	(75)	Pos	12/52 (23)
			Neg	40/52 (77)

\* Pos, Positive delayed hypersensitivity response to DNCB.

† Neg, No delayed hypersensitivity response to DNCB.

scribed the impairment of delayed hypersensitivity reactions to topical application of DNCB in patients with untreated Hodgkin's disease of all stages (37). Only 27% of untreated patients responded to challenge with 100  $\mu$ g of DNCB as compared to 84% of normal individuals. A similar impairment was observed in untreated patients in the present study. Only 36/112 (32%) of the untreated patients in all stages of disease responded to challenge with DNCB. There was no correlation between the absolute number of peripheral blood T lymphocytes, the in vitro response to PHA, and in vivo reactivity to DNCB (Table IV).

In 69 patients, the response to challenge with 100  $\mu$ g DNCB was tested both before treatment and at 3-6 mo intervals after treatment until a positive reaction became apparent. Of 17 patients who demonstrated a positive response before treatment, 15 (88%) were found to be anergic to DNCB immediately after completion of radiotherapy. Nine of the latter regained their capacity to respond to the chemical 4-12 mo after treatment and another patient did so 40 mo after radiotherapy. However, five patients (29%) did not regain their ability to respond to DNCB during a period of up to 8 yr after completion of treatment (Table V).

## DISCUSSION

Most of the reported studies on the effects of ionizing radiation on immune functions in mammals have dealt with single, whole body radiation exposures (38, 39). There have been only a few studies on the effects of localized or regional fractionated irradiation, such as employed in clinical radiotherapy, on immune functions in man. In this study, we have described some of the acute and chronic changes in the number and functions of T and B lymphocytes occurring after TLI of patients with Hodgkin's disease.

Before radiotherapy, the total lymphocyte counts and



the percentage of B cells, T cells, and monocytes were not significantly different from those observed in normal donors. However, at the completion of treatment, the mean total lymphocyte count of 503/mm<sup>3</sup> was 4 SD below the mean for normal controls. Acute lymphocytopenia after a course of radiation has been described in a variety of clinical situations involving localized or extended field radiotherapy (1-20). Recovery usually begins shortly after completion of treatment (6, 7, 12), and continues through the next 1-2 yr. In the present series of treated patients, total lymphocyte counts were restored to pretreatment levels in less than 2 yr. However, there were sustained alterations in the relative frequencies of the various subclasses of lymphocytes with a striking inversion of the normal T vs. B lymphocyte ratio. The percentage of T cells by either E rosette method or the cytotoxicity assay was very low, while the percentage of Ig-bearing cells was significantly increased (Tables I, II). Furthermore, when the combined percentage of T and B lymphocytes was computed, there was a significantly increased percentage of "null" lymphocytes in the treated patients. These changes persisted as long as 8-10 yr in some patients.

Engeset et al. (16) have described an increased percentage of Ig-bearing lymphocytes in 6 out of 7 patients tested 5-36 mo after completion of total lymphoid irradiation for Hodgkin's disease. Cohnen et al. (40) noted a normal percentage of Ig-bearing lymphocytes in four untreated patients with Hodgkin's disease, while the percentage in two other patients treated with radiation and chemotherapy was significantly increased. A postradiotherapy increase in the percentage of complement receptor lymphocytes has also been reported in 40 patients with carcinoma of the breast (1).

Our study suggested that in the acute phase, during fractionated irradiation, there is a severe depletion of the pool of circulating lymphocytes, but after the completion of treatment, there is a gradual restoration of this pool possibly by B-cell precursors originating in the unirradiated bone marrow. The process of restoration seems to be complete by the 1st yr, but for unknown reasons the level of B cells continues to rise for many years, resulting in an "overshoot" in the absolute numbers of B lymphocytes in the circulating pool. This may be a compensatory rise related to the prolonged T lymphocytopenia.

The Ig-bearing cells (B cells) identified in the present study may have acquired surface immunoglobulin either by endogenous production or by binding of exogenous immunoglobulin via Fc receptor. To identify only those B cells with endogenously produced surface immunoglobulin, lymphocytes were incubated *in vitro* for 12 or more h in fetal calf serum at 37°C before staining for surface Ig (35, 36). Incubated lymphocytes from

both normal donors and treated patients showed significant decreases in the percentage of Ig-bearing cells. However, the mean percentage of Ig-bearing cells in treated patients (26.4%) was still more than twofold higher than that in the normal donors (11.7%) (Table III). It is still possible that the increased percentages of Ig-bearing cells postincubation do not represent only B cells, but are partially due to specific antibodies directed against cell surface components of cells other than B cells, which are not removed during the overnight incubation.

Diminished percentages and total numbers of peripheral blood T lymphocytes have previously been reported in patients receiving radiation for carcinoma of the breast (1, 6) and Hodgkin's disease (19, 41). The relative and absolute T lymphocytopenia described in the present report are striking because of the magnitude and the persistence of the changes. Severe depletion of T lymphocytes postirradiation was detected by both the cytotoxicity and the E rosette assays. We have recently reported (31), and have confirmed (Table III) that in untreated patients with Hodgkin's disease, the percentage of T lymphocytes by the E rosette assay is significantly lower than that detected by the cytotoxicity assay. However, this functional impairment is reversed by overnight incubation of the lymphocytes in medium containing 20% fetal calf serum, with restoration of the percentage of E rosette-forming cells up to the level detected by the cytotoxic antibody assay.<sup>2</sup> In contrast, overnight incubation of Ficoll-purified lymphocytes from treated patients in 20% fetal calf serum resulted in no significant change in the percentage of E rosette-forming cell. This suggests that, in contrast to untreated patients, the low percentage of E rosettes observed after radiation therapy reflects a true depletion of T lymphocytes in the peripheral blood.

The nature of the increased population of "null" lymphocytes in treated patients is still unknown. It is not clear whether they represent immature forms of B cells, T cells, or both. It appears, however, that they do not belong to the monocyte group since they did not react in the staining procedure for nonspecific esterases with alpha-naphthol-acetate.

The extensive changes in the numbers of circulating B and T lymphocytes during and after a course of fractionated radiation are also accompanied by functional sequelae. A radiation induced decrease in the response of peripheral blood lymphocytes to PHA has been reported by other investigators in patients receiving various forms of radiotherapy for malignant diseases (2-4,

<sup>2</sup> Fuks, Z., S. Strober, D. P. King, and H. S. Kaplan. 1976. Reversal of cell surface abnormalities of T lymphocytes in Hodgkin's disease after *in vitro* incubation in fetal sera. *J. Immunol.* In press.

6, 8–10, 12–14). In the present series the levels of stimulation of peripheral blood lymphocytes by PHA were significantly lower in treated patients than in patients with untreated Hodgkin's disease, although the responsiveness in the latter group was significantly reduced as compared to normal individuals (Fig. 1). The mechanism of the impaired response to PHA in the treated and untreated groups may be different, since the number of T cells in the peripheral blood of treated patients is significantly reduced as compared to that in untreated patients. It is likely that intensive extended field or total lymphoid irradiation contributes to the decreased response to PHA by eliminating subsets of T cells capable of responding to PHA. If so, our data indicate that there is little, if any, restoration of this subpopulation in the circulating pool during the first 10 yr after therapy. On the other hand, the impaired response in untreated patients appears to be due to a factor which interferes with the capacity of the circulating lymphocytes to form E rosettes and to respond adequately to PHA.<sup>2</sup> We have recently been able to demonstrate such a factor in the serum and in extracts from the spleens of untreated patients with Hodgkin's disease,<sup>3</sup> and to reverse the impaired responses of peripheral blood lymphocytes from these patients by overnight incubation in 20% fetal calf serum.<sup>2</sup>

Lymphocyte stimulation by Con A was also impaired in both untreated patients and in patients in long term remission after radiotherapy (Fig. 2). However, the degree of impairment of the Con A response in the two groups of patients was not significantly different, in contrast to the PHA responses. The possibility that different subpopulations of T lymphocytes may be involved in the response to these two mitogens, as has been suggested by studies in rodents (42), may account for the difference in the levels of stimulation induced by PHA and Con A in treated patients in the present study.

The in vitro proliferative response to recall antigens, such as tetanus toxoid, and to allogeneic lymphocytes seems to be specific to T cells alone, even when examined in long term (6 day) cultures (43). The suppressed response to tetanus toxoid in untreated patients is probably related to altered T-cell function, since these patients generally do not have T lymphocytopenia (31). However, after radiation, the decreased response could well be attributable to both decrease in number and impairment of function of T cells, since increasing the number of tested cells from treated patients from 200,000 to 400,000 did not restore their response (Fig. 5).

<sup>2</sup> Fuks, Z., S. Strober, and H. S. Kaplan. 1976. Interaction between serum factors and T lymphocytes in Hodgkin's disease: Use as a diagnostic test. *N. Engl. J. Med.* In press. Bieber, M. M., Z. Fuks, H. S. Kaplan, and C. P. Bieber. 1976. E rosette inhibiting substance in Hodgkin's disease spleen extract. Submitted for publication.

The finding that peripheral blood lymphocytes from patients with untreated Hodgkin's disease react normally when used as a source of responder cells in the MLR test (Fig. 4) is consistent with other reports in the literature (44, 45). After total lymphoid irradiation, there was a marked reduction of the ability of the peripheral blood lymphocytes to respond in the MLR reaction (Fig. 5). This persisted for at least 2 yr after irradiation, but a partial and progressive recovery occurred during the 3rd through the 5th yr after treatment. Normal MLR responses are observed in most patients in complete remission for 5 yr or more.

A similar pattern of recovery from radiation damage exists for the delayed hypersensitivity response to DNCB, in those patients who were sensitive to the allergen before TLI. It is also of interest that some patients who were anergic to DNCB before treatment became reactive to the allergen after radiotherapy (Table V). It is conceivable that the repeated challenges with 100  $\mu$ g of DNCB served to sensitize such patients after their disease had been eradicated. It has been shown that a single application of 100  $\mu$ g DNCB can induce delayed hypersensitivity in 19% of normal individuals (34, 37). An alternative hypothesis is that sensitization to the chemical did indeed occur before treatment despite the fact that Hodgkin's disease activity prevented the normal response to challenge, and that a population of specific memory precursors which survived in these patients throughout irradiation later provided effector cells for the hypersensitivity response after active disease had been eradicated. The recovery of the response in these patients is considerably slower than that of patients who show hypersensitivity to DNCB before radiotherapy.

The recovery from radiation damage observed for the hypersensitivity response to DNCB and for the MLR test is in marked contrast to the lack of recovery of the responses to PHA and Con A which persists for at least 10 yr. It is possible that the differences in the kinetics of recovery of the different tests reflect differences in both the nature of the mitogenic stimulus and in the in vitro assays, since the MLR response was measured by  $H^3$  TdR uptake and the PHA and Con A response by [ $H^3$ ]leucine uptake. The initial general elimination of T-cell functions correlates with the depletion of the pool of circulating T cells during a course of radiotherapy. The slow rates of postradiation recovery of these functions suggest that the process of T-cell maturation may be impaired after total lymphoid irradiation. Some subsets of partially differentiated lymphocytes may fail to complete the normal thymus-dependent sequence of morphological and functional maturation due to the lack of thymic influence caused by normal age-dependent thymic involution and by irradiation. Other factors

such as splenectomy, destruction of the normal matrix of lymph nodes by radiation, and perhaps the production of specific anti-T-antibodies may also participate in the process of maturation arrest. Variations of the initial radiosensitivity of different subpopulations of T cells and in the rates of their maturation may also contribute to the eventual pattern of recovery after irradiation.

The relatively slow recovery of cell mediated immunity after irradiation indicated that total lymphoid irradiation may be a potent modality for the induction of long term immunosuppression. The lack of MLR response during the first 2 yr after radiotherapy, and its slow recovery over the following 2-3 yr indicates that total lymphoid irradiation may prolong the survival of allografts. Indeed, recent preliminary experiments in our laboratory have shown that total lymphoid irradiation by a technique similar to that employed in clinical radiotherapy prolongs the survival of allogeneic skin transplants in mice.<sup>4</sup> The clinical utilization of total lymphoid irradiation as a mode of immunosuppression in patients receiving organ transplants is a provocative idea, but extensive additional studies will be necessary before the merits of such an approach can be fully assessed.

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<sup>4</sup> Slavín, S., S. Strober, Z. Fuks, and H. S. Kaplan. Long term survival of skin allografts in mice given fractionated total lymphoid irradiation. *Science (Wash. D. C.)*. In press.

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