Depressed In Vitro Lymphocyte Responses to PHA in Patients With Hodgkin Disease in Continuous Long Remissions

By Delvyn C. Case, Jr., John A. Hansen, Ernestina Corrales, Charles W. Young, Bo Dupont, Carl M. Pinsky, and Robert A. Good

Twenty consecutive patients with Hodgkin disease in continuous complete remission and off treatment for at least 5 yr (range 5–25 yr, median 9 yr) were studied with a battery of immunologic parameters. Skin test reactivity to four common antigens, sensitization to 2,4-dinitrochlorobenzene, absolute lymphocyte count, relative percentage of T cells (as measured by spontaneous rosette formation with sheep erythrocytes) and B cells (as measured by immunofluorescence with polyclonal antisera), and absolute number of T and B cells were normal when compared with controls. However, the mean value of lymphocyte response in vitro to the mitogen phytohemagglutinin for the study population was significantly decreased (p < 0.001) when compared with the controls. This abnormality in response to mitogen could not be correlated with age, sex, stage, symptoms, histologic subclassification, or previous treatment. The data suggest the existence of a persisting cell-mediated immune defect in the circulating lymphocytes in patients with long-standing Hodgkin disease that might otherwise be considered "cured."

Earlier studies of the immunologic reactivity of Hodgkin disease have demonstrated abnormalities in the absolute lymphocyte count,1 absolute number of T cells,2 and lymphocyte function;3,4 but these reports have included many patients previously undergoing treatment. Recently, in a large group of untreated patients, we have found that the absolute lymphocyte count and absolute number of T and B cells are decreased while the relative number of T and B cells and the T and B cell ratio are normal.5 The absolute T cell number and percentage of T cells, however, have been reported as normal by Bobrove et al.6 Significant abnormalities in response to 2,4-dinitrochlorobenzene (DNCB) sensitization,5–8 lymphocyte response to phytohemagglutinin (PHA),5,9,10 and lymphocyte response to specific antigens in vitro11 have also been documented in untreated patients. The degree of immunologic recovery of such patients following effective therapy is of current interest. Aisenberg12 has found return of DNCB sensitivity in patients in remission for longer than 2 yr; but Levy and Kaplan,10 using a more sensitive assay, have reported that less than 50% of patients who had been in remission for up to 10 yr can be sensitized to DNCB. Han and Sokal3 have reported a normal in vitro lymphocyte response to PHA in 30 patients studied during remission of greater than 1 yr. In
contrast, Levy and Kaplan have found that the mean PHA response in a seemingly comparable group of patients has remained abnormal despite continuous remission for 2–8 yr.

This report supplements the available information by recording the results of immune testing of 20 previously treated patients with Hodgkin disease in continuous complete remission and off all therapy for at least 5 yr. We have surveyed the following six measurements of immune competence in these patients: (1) skin testing with four common microbial antigens; (2) DNCB sensitization with a standard dose (2000 μg); (3) lymphocyte transformation in response to several mitogens [PHA, pokeweed mitogen (PWM), and concanavalin A (Con A)]; (4) absolute number of lymphocytes; (5) absolute number and ratio of T to B cells; and (6) immunoglobulin levels. The results of immunologic studies in this group of apparently disease-free patients suggest a return of lymphocyte number but persistence of an abnormality of lymphocyte function as measured by testing in vitro.

MATERIALS AND METHODS

Patients
The subjects of this study were 20 consecutive patients with Hodgkin disease in continuous complete remission and off all therapy for at least 5 yr who returned for routine follow-up examinations at Memorial Hospital between October 1974 and May 1975. Care was taken that none of the patients had other intercurrent medical problems or infections. Prior to immunologic study, the patients were evaluated with a careful physical examination, complete blood count (CBC) and differential, liver function tests, and chest x-ray. The histologic diagnosis of Hodgkin disease was reconfirmed, and subtypes identified according to the Rye modification of the Lukes and Butler classification.

Skin Testing
All patients were skin tested with a battery of intradermal antigens, including purified protein derivative of tuberculin (PPD), mumps, Candida albicans, and streptokinase-streptodornase. If a patient did not react to either the intermediate-strength PPD or 5 units of streptokinase-streptodornase, he would be rechallenged with a higher dose (second-strength PPD, 250 units) or 50 units of streptokinase-streptodornase. The tests were read at 48 hr and considered positive if the diameter of induration was more than 5 mm. Anergy was defined as the lack of response to all four antigens. In addition, patients were tested for the ability to become sensitized to DNBC at a sensitizing dose of 2000 μg DNBC in 0.1 ml acetone as previously described. Rarely did normal controls or patients in this study demonstrate de novo sensitivity to this dose at 48 hr. Prior hypersensitivity to DNBC was excluded by control testing with 100 μg, 50 μg, and 25 μg DNBC in 0.1 ml acetone applied simultaneously to the ipsilateral forearm and examined at 48 hr. In 14 days, challenge doses of 100 μg, 50 μg, and 25 μg DNBC in 0.1 ml acetone were applied to the ipsilateral forearm. The reaction was considered positive if the area of induration was 5 mm or more in 48 hr. There was only an occasional response to the 50-μg and 25-μg challenge doses, and the data reported here are the results of the 100-μg challenge.

In our control series, 90%–100% of normal individuals responded to at least one of the common antigens, just as most normal individuals can be sensitized to DNBC by the method used in this study.

Lymphocyte Transformation
Lymphocyte transformation in vitro was performed using a micro method. Lymphocytes were isolated from heparinized blood on Ficoll-Hypaque (Lymphoprep) density gradients and resuspended in RPMI 1640 with 25 mM Hepes buffer, penicillin (10 U/cm), streptomycin (10 U/cm), L-glutamine (0.25 mg/cm), heparin (10 IU/cm) and 15% pooled normal human serum. Lymphocyte cultures were performed in two different ways: (1) 100,000 lymphocytes
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cultured in 200 µl media in flat-bottomed microtiter plates (No. 3040, Falcon Plastics, Oxnard, Calif.), or (2) 50,000 lymphocytes cultured in 150 µl media in round-bottomed microtiter plates (Cooke No. 1-221-24-1, Cooke Laboratory Products, Alexandria, Va.). Stimulation was performed using phytohemagglutinin (PHA-P, Difco Laboratories, Detroit, Mich.), Con A (Difco), and PWM (Gibco, Grand Island, N.Y.) Dose–response curves were determined with each of the three mitogens. Dilutions of each mitogen were prepared in advance and aliquots stored at -20°C. Six concentrations of PHA were used ranging from 250 to 2.5 µg/ml. Six concentrations of Con A were used ranging from 83 to 6.7 µg/ml. Three concentrations of PWM were used: 1:10, 1:50, and 1:250 dilutions.

25 µl of freshly thawed mitogen was added to each culture and each culture was performed in triplicate. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Mitogen-stimulated cultures were incubated for 72 hr, and antigen-stimulated cultures were incubated for 120 hr.

Labeling was then performed by adding 25 µl of 14C-thymidine (0.03 µCi) to each culture. Labeling was continued for another 24 hr of incubation and then the cultures were removed for harvesting on a multiple-sample machine (Skatron). The cells were collected and washed semi-automatically on glass-fiber filters, dried, and then transferred to scintillation vials for counting. The average proliferation in stimulated triplicate cultures was expressed as net counts per minute (cpm).

The studies on these 20 patients were performed over a period of 9 mo. The control group used to define the normal in vitro response to mitogen consisted of 46 different normal blood donors (ages 20–50 yr old) who were tested concurrently with the patients.

**Lymphocyte Count, T- and B-Cell Determination**

The absolute lymphocyte count was derived from the total white blood cell count (Coulter counter) and the differential count. For the 17 normal blood donors who were studied on the same day as the patients, the mean absolute lymphocyte count was 1930/cu mm with a range of 752–3486/cu mm.

Lymphocytes for the determination of T and B cells were isolated from blood on Ficoll-Hypaque (Lymphoprep) density gradients. The lymphocyte fraction was washed three times in Hank’s balanced salt solution and then incubated with latex particles for 1 hr at 37°C to assist in the identification of monocytes. The number of spontaneous rosette-forming cells (T cells) was determined, with minor modifications, by the method of Jondal et al. An aliquot of lymphocyte suspension was incubated with sheep erythrocytes in 10% absorbed human AB serum and the number of cells forming erythrocyte rosettes was counted after 18 hr of incubation at 4°C. An aliquot of lymphocyte suspension for determination of B cells was washed twice in Hank’s balanced salt solution following incubation with latex particles and then resuspended in 2% bovine serum albumin in phosphate-buffered saline with 0.2% sodium azide. Immunofluorescent staining with anti-immunoglobulin was performed using a fluoresceinated polyvalent antiserum (Dako-Immunoglobulins, Copenhagen, Denmark). The number of non-latex-ingesting surface-staining B cells was determined using fluorescent microscopy as previously described.

The relative percentage of T cells in the peripheral blood as measured by spontaneous rosettes in the 17 controls was 82.4% ± 8%, while the mean absolute number of T cells in the peripheral blood was 1320/cu mm, with a range of 541–2754/cu mm. The relative percentage of B cells in the normal controls as measured by this technique for direct membrane immunofluorescence was 14.3% ± 8%. The mean absolute number of B cells was 283/cu mm, with a range of 98–1116/cu mm.

**Immunoglobulin Determination**

Quantitation of serum immunoglobulins was performed by the single radial immunodiffusion technique using commercial plates (Hyland Division, Travenol Laboratories, Costa Mesa, Calif.).

**RESULTS**

In Table 1 the histologic subtypes and stages of the 20 patients in this study are shown. Table 2 presents the clinical characteristics of the patient group. There were 13 males and 7 females; 16 were between the ages of 9 and 35 at the
time of diagnosis, four were older than 35, and the mean age was 26. The most common histologic subtype was nodular sclerosing: 17 patients were IA–III A; only 3 patients had “B” symptoms. The range of continuous complete remissions was 5–25 yr with a median of 9 yr. Sixteen of the patients were treated with radiotherapy alone; three had both radiotherapy and chemotherapy. Only one patient had chemotherapy alone. In the radiotherapy group, each patient was treated with approximately 3500 rads to the areas radiated: 11 patients received a mantle port; 5 patients received a mantle port plus inverted Y; and 3 patients received radiotherapy to a local cervical port for IA disease.

Table 1. Distribution of Histologic Subtypes

<table>
<thead>
<tr>
<th>Stage</th>
<th>LP</th>
<th>NS</th>
<th>MC</th>
<th>LD</th>
<th>Total Patients in Each Stage</th>
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<tr>
<td>I</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>0</td>
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<td>III</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

Abbreviations: LP, lymphocyte predominant; NS, nodular sclerosing; MC, mixed cellularity; LD, lymphocyte depleted.

Table 2. Clinical Characteristics and Relation to Immune Parameters

<table>
<thead>
<tr>
<th>Age (yr) at Onset/Sex</th>
<th>Stage/Histology</th>
<th>Treatment</th>
<th>Time After Treatment (yr)</th>
<th>Skin Tests</th>
<th>DNBC</th>
<th>Lymphocyte Count (cells/cu mm)</th>
<th>PHA Response (cpm)</th>
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</thead>
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<tr>
<td>16/F IIIA/NS RT(M)</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>1983</td>
<td>33,935</td>
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<td></td>
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<td>44/M IIIA/MC Bulsulphan</td>
<td>21</td>
<td>–</td>
<td>+</td>
<td>2200</td>
<td>27,299</td>
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<tr>
<td>18/F IA/NS RT(M)</td>
<td>5</td>
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<td>+</td>
<td>1482</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>2147</td>
<td>25,082</td>
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<tr>
<td>30/M IIIA/NS RT(M) +</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>1719</td>
<td>24,335</td>
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<td></td>
<td>vinblastine</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32/M IA/IL RT(L)</td>
<td>24</td>
<td>+</td>
<td>+</td>
<td>2200</td>
<td>22,643</td>
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<td>16/F IIIA/MC RT(M)</td>
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<td>+</td>
<td>+</td>
<td>1848</td>
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<td>36/F IIIA/MC RT(M)</td>
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<td>+</td>
<td>3774</td>
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<tr>
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<td>30/M IA/NS RT(L)</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>9/M IIIA/NS RT(M)</td>
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<td>+</td>
<td>+</td>
<td>800</td>
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<tr>
<td>21/M IA/NS RT(M) +</td>
<td>6</td>
<td>+</td>
<td>–</td>
<td>1330</td>
<td>18,562</td>
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<tr>
<td></td>
<td>vinblastine + chlorambucil</td>
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<td></td>
<td></td>
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<tr>
<td>20/M IIIA/NS RT(M)</td>
<td>9</td>
<td>+</td>
<td>+</td>
<td>2000</td>
<td>17,135</td>
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<td>12/F IIIB/NS RT(M)</td>
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<td>+</td>
<td>+</td>
<td>436</td>
<td>15,786</td>
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<tr>
<td>21/M IIIIB/MC RT(M + Y) + vinblastine + chlorambucil</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>1885</td>
<td>15,169</td>
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<tr>
<td>23/F IA/NS RT(M)</td>
<td>7</td>
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<td>+</td>
<td>1529</td>
<td>15,107</td>
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<tr>
<td>20/M IIIA/NS RT(M + Y)</td>
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<td>+</td>
<td>3300</td>
<td>24,019</td>
<td></td>
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<tr>
<td>44/M IIIA/MC RT(M + Y)</td>
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<td>+</td>
<td>1531</td>
<td>13,820</td>
<td></td>
<td></td>
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<tr>
<td>40/F IA/IL RT(L)</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>483</td>
<td>13,523</td>
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+, normal; –, abnormal; †, abnormally low; RT, radiotherapy; M, mantle; Y, inverted Y; L, local.

*Mean maximum PHA response for the normal control group was 27,539 cpm (SD 5345 cpm). An abnormal PHA response was defined as a response less than 16,849 cpm (mean – 2 SD).
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After 5 yr off treatment and in continuous complete remission, 90% of the patients in this study demonstrated skin-test reactivity to common antigens and sensitization to DNCB with the 2000 μg dose. These results were not significantly different from the normal control group.

The mean absolute lymphocyte count for the patient group was 1803/cu mm with a range of 436–3774/cu mm. The mean absolute number of T cells in the peripheral blood of patients was 1327/cu mm (range 348–2936/cu mm) and the mean absolute number of B cells was 248/cu mm (range 0–906/cu mm). Figure 1 illustrates the absolute numbers of total lymphocytes, T cells, and B cells for the 20 patients and the 17 normal controls. No significant difference in the mean values for total lymphocytes, T cells, or B cells could be found between the normal controls and patients with prolonged continuous remissions. The relative percentage of T and B cells and the T-to-B cell ratios were also normal in the patient group.

For the 20 patients studied, the mean maximum response to PHA was 20,749 cpm (median 19,830 cpm) with a range of 13,523–33,935 cpm (SD 5097 cpm). The mean maximum response for the group of 46 normal controls was 27,539 cpm with a range of 18,604–38,229 cpm (SD 5345 cpm). The maximum responses to PHA for the 20 patients and 46 normal controls are shown in Fig. 2. The mean response of the study group was significantly lower than that of the controls (Student's t test, p < 0.001). Patients in this study were in continuous remission and off treatment for 5–25 yr (median 9 yr). This abnormal response to PHA could not be correlated with age at presentation, sex, stage, symptoms, histologic subclassification, or treatment (Table 2). Four patients underwent splenectomy. All four had lymphocyte responses to PHA above the mean for the patient group.

Lymphocyte response to PWM and Con A were also measured. In 85% of patients and controls, the lymphocyte response to these mitogens paralleled the response to PHA. Dose–response curves for lymphocyte stimulation to PHA were analyzed to determine if the pattern of transformation to different concen-
trations of mitogen would provide a curve that would discriminate this study population from normals. No additional information was provided by the dose–response curves. By differences in the maximum response, the patient group could be distinguished from the control group.

Immunoglobulin levels in these patients were found to be within the normal range.

**DISCUSSION**

Studies in untreated patients with Hodgkin disease have shown abnormalities in both lymphocyte number and function, but the reconstitution of the immune system in patients in prolonged complete remission is still unclear. Aisenberg reported that 11 of 12 patients in remission for longer than 2 yr could be sensitized to DNCB. Levy and Kaplan, however, found that less than 50% of patients in remission followed for up to 10 yr had a positive reaction to DNCB. Han and Sokal studied 30 patients in remission for more than 1 yr and found that all had normal lymphocyte responses to PHA regardless of initial stage, histologic subtype, skin-test results, lymphocyte count, and duration of disease. They also found that radiotherapy consistently depressed lymphocyte response to PHA but recovery occurred in 1–3 mo. Another group found return of normal lymphocyte response to PHA 6 mo after radiotherapy or chemotherapy. However, the Stanford group found the mean PHA response depressed in a group of patients treated with radiotherapy who were disease-free for 2–8 yr.

In this report we have studied 20 patients in continuous remission from Hodgkin disease and off treatment for 5–25 yr (median 9 yr). The absolute number of lymphocytes and the subpopulations of T and B cells (percentage and absolute number) were found to be normal, along with skin-test reactivity to intradermal antigens, sensitization to DNCB, and immunoglobulin levels. Lymphocyte function in vitro as measured by response to PHA, however, was found to be significantly impaired (p < 0.001). This impairment was not found to correlate with lymphocyte count, skin-test reactivity, DNCB sensitization, age, sex, stage, symptoms, or histologic subtype.

The abnormality in lymphocyte number and function as defined in untreated patients and the data presented in this paper suggest that the total lymphocyte number and the subpopulations of T and B cells may return to normal levels in long-term survivors but that deficient lymphocyte function as measured by in vitro response to PHA can persist.

The pathophysiology of this impaired response to in vitro mitogens is yet unclear. It may be a result of long-lasting impairment of T-cell function (1) by the original neoplastic process itself, (2) by the effects of treatment, or (3) by persistent subclinical disease. Localized or generalized lymphoid involvement with or without thymic involvement by Hodgkin disease may produce a permanent defect. In our series, seven patients had disease clinically limited to cervical nodes. However, the mean value of response to PHA in this group of IA patients was not significantly different from the entire group of 20 patients, which included patients with stages I–IV. In one series of patients with laryngopharyngeal carcinoma, the lymphocyte response to PHA and the absolute number of T cells were found to be decreased in patients who had received radiotherapy (1200–6000 rads) restricted to the region above the clavicles. These
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Immune defects persisted in patients that were in complete remission for 4–15 yr (mean 9 yr) but were not found in patients that had been treated with surgery. In a series of breast cancer patients treated with radiotherapy (2000–2400 rads) to a unilateral parasternal port, decreased total lymphocyte count, decreased T cells with increased B cells, and decreased response to PHA persisted for at least 1 yr. Depression of absolute number of T cells has also been reported following local radiation for urologic cancers. In our series no statistical difference in response to PHA could be found between the patients receiving local cervical radiotherapy, mantle field, and mantle field plus inverted Y. All the patients received approximately 3500 rads to fields irradiated. These observations suggest that radiation therapy itself might have long-term effects on lymphocyte number and function even when the field did not involve all lymph nodes or the thymus. It is of great interest that persistent Hodgkin disease has been found in long-term survivors succumbing from other causes without any apparent clinical Hodgkin disease. Such subclinical disease might contribute to abnormal lymphocyte function. No patient in this study has relapsed or expired from other causes to date.

The identification of those patients with persisting immune defects in prolonged remissions might be clinically important. Impaired lymphocyte function as measured by response to PHA, as shown in this study and by the Stanford group, might be related to the risk of late recurrences or second primaries seen after successful treatment of Hodgkin disease.

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