Abnormal Phytohemagglutinin-Induced T-Cell Proliferative Responses in Hodgkin's Disease

By Richard S. Schulof, Mortimer J. Lacher, and Sudhir Gupta

Optimal conditions were established for evaluating the phytohemagglutinin-induced proliferative responses of purified peripheral blood T lymphocytes. This assay was utilized to determine whether T cells (in the absence of monocytes and serum inhibitory factors) from patients with Hodgkin's disease were defective in their ability to proliferate in response to optimal (50 μg/ml) and suboptimal (25 and 12.5 μg/ml) concentrations of phytohemagglutinin. T cells from 6 of 12 untreated patients exhibited 6-day proliferative responses below the range of 15 control subjects using optimal mitogen concentrations, and 9 of 12 patients exhibited subnormal responses using lower concentrations. Kinetic analyses indicated that the abnormal T-cell proliferative responses were characterized by peak proliferation occurring at day 4 or 5, rather than day 6. The observed abnormalities were not related to elevations in the proportions of T cells bearing surface receptors for IgG (Tγ Cells). Our results suggest that intrinsic functional T-cell defects contribute to the impaired immunity associated with Hodgkin's disease.

PATIENTS with Hodgkin's disease (HD) frequently exhibit defective cell-mediated immune responses, such as impaired lymphoproliferative responses (LPR) to the T-cell mitogen, phytohemagglutinin (PHA). Several factors contribute to the depressed PHA-reactivity, including the presence of (1) circulating suppressor monocytes, (2) monocyte-derived inhibitory products (e.g., prostaglandins), and (3) immunosuppressive serum substances. Although it is clear that exogenous factors can influence T-cell mitogenesis in HD, it has not been determined whether T cells, in the absence of monocytes and serum inhibitors, are still defective in this ability.

For the past several years, we have been characterizing the T-cell defects associated with primary and secondary immunodeficiency disorders. In our studies we have utilized peripheral blood T lymphocytes as well as T-lymphocyte subpopulations. We have previously shown that patients with HD exhibit quantitative abnormalities in the proportions of circulating T-cell subsets (Tγ, Tμ) and functional T-cell abnormalities, such as impaired locomotor activity. It has also become apparent that monocyte-free T cells and purified T-cell subpopulations are capable of undergoing PHA-induced proliferation. In the present investigation, we have determined the optimal conditions for evaluating T-cell LPR (T-LPR) to PHA. In addition, we have found that 10 of 12 patients with HD exhibited abnormalities in their T-LPR. Our results provide further evidence to indicate that intrinsic T-cell functional defects contribute to the impaired immunity associated with HD.

MATERIALS AND METHODS

Subjects

Control subjects were healthy blood bank donors. T cells prepared from the normal subjects were used for assay optimization and as controls for the patients with Hodgkin's disease. Twelve previously untreated patients with Hodgkin's disease were studied prior to staging laparotomy. Tissue biopsies were histologically subclassified according to the Rye system. The majority of patients underwent staging laparotomy and were pathologically staged according to the Ann Arbor classification. The patient population included 7 males and 5 females, whose ages ranged from 9 to 52 yr (median, 28 yr). All but one of the patients were clinically or pathologically stage I or II. Other patient characteristics are shown in Table I. Fifteen control subjects included 9 males and 6 females, ranging in age from 23 to 55 yr (median, 31 yr).

Preparation of T Cells

T cells were prepared from fresh heparinized blood as previously described. Mononuclear cells were obtained by Ficoll-Hypaque (FH) density gradient centrifugation. Interface cells were washed three times and resuspended (4 x 10⁶/ml) in complete medium [CM: RPMI 1640 + 20 mM Heps + 100 U/ml penicillin + 100 μg/ml streptomycin + 2 mM L-glutamine + 20% fetal calf serum (FCS), Gibco, Grand Island, N.Y.. Lot A193712]. Phagocytic cells (monocytes and residual granulocytes) were removed by incubation with one-half volume carbonyl iron reagent (Lymphocyte Separator Reagent, Technicon, Tarrytown, N.Y.) at 37°C for 1 hr, followed by recentrifugation on FH. The monocyte-depleted lymphocytes were utilized for analysis of E-rosette-forming cell (E-RFC) percentages. T cells were isolated by E-rosette formation under optimal conditions employing neuraminidase-treated sheep erythrocytes (nSRBC) and including 25% FCS (previously absorbed with SRBC) in the incubation mixture. Equal volumes of lymphocyte suspensions (4 x 10⁶/ml) in balanced salt solution were mixed with 1% nSRBC and one-quarter volume FCS. Cells were incubated for 5 min at 37°C, centrifuged at 200 g for 5 min, and incubated for 1 hr at 4°C. Pellets were then gently resuspended, and rosetted T cells were isolated from non-T-cells by FH centrifugation, followed by lysis of erythrocytes with Tris buffer (pH 7.2) containing 0.83% ammonium chloride. T cells were incubated overnight in CM at 37°C prior to use. Cell viability, assessed by trypan blue dye...
Table 1. Characteristics of Patients With Hodgkin’s Disease

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/Sex</th>
<th>Stage</th>
<th>Histology</th>
<th>Percent T Cells (E-RFC)</th>
<th>Abs. Lymph/μm²</th>
<th>Abs. T Cells/μm²</th>
<th>Percent T1</th>
<th>Percent T2</th>
<th>Percent Peroxidase</th>
<th>6-Day T-Cell PHA Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28 M</td>
<td>I-E</td>
<td>Unc</td>
<td>76%</td>
<td>837</td>
<td>636</td>
<td>34%</td>
<td>12%</td>
<td>2.8</td>
<td>0.2%</td>
</tr>
<tr>
<td>2</td>
<td>9 M</td>
<td>II-A</td>
<td>NS</td>
<td>75%</td>
<td>2,299</td>
<td>1,724</td>
<td>46%</td>
<td>8%</td>
<td>5.8</td>
<td>0.6%</td>
</tr>
<tr>
<td>3</td>
<td>37 F</td>
<td>II-A</td>
<td>NS</td>
<td>78%</td>
<td>2,698</td>
<td>2,096</td>
<td>ND**</td>
<td>ND</td>
<td>6.5</td>
<td>0.1%</td>
</tr>
<tr>
<td>4</td>
<td>28 M</td>
<td>II-A</td>
<td>NS</td>
<td>71%</td>
<td>910</td>
<td>646</td>
<td>58%</td>
<td>13%</td>
<td>4.5</td>
<td>0.1%</td>
</tr>
<tr>
<td>5</td>
<td>39 M</td>
<td>II-A</td>
<td>NS</td>
<td>83%</td>
<td>730</td>
<td>608</td>
<td>32%</td>
<td>31%</td>
<td>1.0</td>
<td>0.15%</td>
</tr>
<tr>
<td>6</td>
<td>21 F</td>
<td>II-A</td>
<td>NS</td>
<td>85%</td>
<td>1,080</td>
<td>918</td>
<td>ND</td>
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<tr>
<td>7</td>
<td>29 F</td>
<td>III-A</td>
<td>Unc</td>
<td>81%</td>
<td>2,925</td>
<td>2,345</td>
<td>46%</td>
<td>8%</td>
<td>5.1</td>
<td>0.1%</td>
</tr>
<tr>
<td>8</td>
<td>34 M</td>
<td>II-B</td>
<td>NS</td>
<td>82%</td>
<td>2,888</td>
<td>2,196</td>
<td>41%</td>
<td>8%</td>
<td>1.0</td>
<td>0.1%</td>
</tr>
<tr>
<td>9</td>
<td>32 F</td>
<td>III-A</td>
<td>Unc</td>
<td>87%</td>
<td>1,530</td>
<td>1,241</td>
<td>30%</td>
<td>21%</td>
<td>1.4</td>
<td>0.1%</td>
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<tr>
<td>10</td>
<td>5 M</td>
<td>III-A</td>
<td>NS</td>
<td>71%</td>
<td>1,430</td>
<td>1,018</td>
<td>25%</td>
<td>24%</td>
<td>1.0</td>
<td>0.3%</td>
</tr>
<tr>
<td>11</td>
<td>17 F</td>
<td>III-A</td>
<td>NS</td>
<td>74%</td>
<td>1,378</td>
<td>1,019</td>
<td>25%</td>
<td>24%</td>
<td>1.0</td>
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<tr>
<td>12</td>
<td>18 M</td>
<td>III-A</td>
<td>NS</td>
<td>69%</td>
<td>441</td>
<td>304</td>
<td>48.2</td>
<td>10.6</td>
<td>5.1</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Normal values: 79.5% ± 29, 1901 ± 29, 1508 ± 29, 48.2 ± 29, 10.6 ± 29, 5.1 ± 29

Mean ± SEM: 8.2 ± 1.3, 468 ± 1.3, 392 ± 1.3, 17.4 ± 1.3, 4.9 ± 1.3, 0.6 ± 1.3

n: 6-9, 1.32 ± 0.6, 2.59 ± 0.6, 2.10 ± 0.6, 0.3 ± 0.6, 0.3 ± 0.6

Range: 1.32-3.000, 1.016-2.595, 21.0-78.0, 3.3-17.0, 0.3-0.6

Denotes clinical staging. All other patients were pathologically staged.

†Determined using monocyte-depleted lymphocytes.

‡Percent peroxidase-positive (monocytes and granulocytes) cells contaminating T-cell preparations; 2000 cells were counted. T cells from all subjects contained >95% E-RFC and <1% surface immunoglobulin-bearing cells.

§Considered decreased if <95% E-RFC, <1% E-RFC, <1% surface immunoglobulin-bearing cells, and <0.1% peroxidase-staining cells (monocytes and granulocytes).19

T-Cell Analysis

E-RFC percentages were determined on monocyte-depleted lymphocytes according to the method of Bentwich et al.28 T cells with receptors for IgG (Tγ cells) or IgM (Tμ cells) were determined by EA-rosette formation using IgG or IgM-coated ox-RBC, respectively.14 Absolute T-cell levels were determined by multiplying E-RFC percentages by absolute lymphocyte count determined by Wright-stained blood smears.

PHA Assay

Assays were performed in triplicate in U-bottomed microtiter plates, using 50 x 10³ T cells/well. Assay volumes were adjusted to 220 μl with CM, which included the appropriate concentrations of PHA-P (Difco Labs, Detroit, Mich.). Plates were incubated at 37°C in a humidified environment containing 95% air, 5% CO₂, and were pulsed with 2 μCi ³H-thymidine (New England Nuclear, Boston, Mass.) 16 hr prior to harvesting. Individual wells were harvested onto glass fiber filter paper using an automated sample harvester. After drying, samples were transferred to counting vials, scintillant was added, and samples were counted in a liquid scintillation counter. Data are expressed as net cpm ³H-thymidine incorporated and reflects cpm ³H-thymidine incorporated in the presence of PHA-background cpm (cpm incorporated in the absence of PHA). Background cpm varied from 400 to 1,200 cpm for both patients as well as control subjects.

Statistical analyses were performed using the Student's t test for independent samples.21

RESULTS

Assay Optimization

T cells from healthy subjects were used to determine the optimal conditions for evaluating PHA-induced T-LPR. As seen in Fig. 1, maximal T-LPR required a PHA concentration of at least 25 μg/ml and 6-7 days of incubation. A comparison of LPR using T cells and mononuclear cells (MC) prepared from the same donor is shown in Fig. 2. Peak MC-
cases, T-cell preparations were extremely pure and the percentage of contaminating peroxidase-staining cells (PC: monocytes and granulocytes) ranged from -0.1% to 0.6%.

**T-Cell Proliferative Responses**

T-cell LPRs were evaluated using 3 different PHA concentrations (12.5, 25, and 50 μg/ml) and a 6-day incubation period. With the highest PHA concentration (50 μg/ml), 6 of 12 patients exhibited T-LPRs below the range of 15 control subjects (Fig. 3). At the lower PHA concentrations (25 μg/ml and 12.5 μg/ml), 9 of 12 patients exhibited T-LPRs below the range of the controls. When individual patients were analyzed, 10 exhibited T-LPRs below the normal range using at least two different PHA concentrations (Table 1). As a group, the Hodgkin's patients exhibited significant impairments of T-LPRs at all PHA concentrations used (Table 2). Impaired T-LPRs were not related to the percentage of PC contaminating the T-cell preparations since they were observed in patients with both low (<0.1%) and high (0.6%) PC concentration. Impaired T-LPRs were not related to abnormalities in T-cell subset proportions, and no

**Hodgkin's Patients**

The characteristics of the patients used for T-LPR analyses are shown in Table 1. Peripheral blood lymphocytes (PBL) from all 12 patients exhibited normal percentages of E-RFC, although 6 patients (nos. 1, 4, 5, 6, 10, 12) exhibited absolute T-cell lymphopenia. Of the patients tested, 3 of the 7 (nos. 5, 9, 11) exhibited moderate elevations in $T_\gamma$ cell proportions. $T_\mu$ percentages were within the normal range for all patients studied. However, 4 patients exhibited depressions of the $T_\mu/T_\gamma$ ratio (nos. 1, 5, 9, 11). In all
relationships could be identified between elevated Tγ proportions or depressed Tμ/Tγ ratios and abnormal T-LPRs. In fact, 2 of 3 patients with elevated Tγ percentages exhibited normal PHA-induced proliferation.

**Kinetic Studies**

Time studies (days 3–8) using T cells from 2 patients who exhibited subnormal 6 day T-LPRs and from one patient who exhibited a normal 6 day T-LPR are shown in Figs. 4 and 5. As illustrated in Fig. 4, patients who exhibited normal 6-day T-LPRs also exhibited peak proliferative activity on day 6. In addition, a second peak of activity was seen on day 4, which was not observed with T cells from control subjects. T cells from the HD patients who exhibited subnormal 6-day T-LPRs were found to exhibit only a single peak of proliferative activity, which occurred prior to day 6 (i.e., day 4 or 5, Fig. 5). Thus, such an early peak of proliferative activity was unique to the HD patients regardless of whether or not they exhibited subnormal 6 day T-LPRs.

From the kinetic analysis described above, it was possible that although the day 6 LPRs of HD T cells were depressed, their maximal peak proliferative responses occurring on day 4 might be quantitatively normal when compared to the 6-day LPRs of the controls. To explore this possibility, comparisons were made between day 4 T-LPRs of 6 HD patients and day 4 and day 6 T-LPRs of the normal controls (Table 2). Day 4 represented the maximal T-LPRs of the HD patients and suboptimal T-LPRs for the controls. There were no significant differences between 4-day T-LPRs of the patient and control groups. When day 4 T-LPRs of the HD patients were compared to day 6 T-LPRs of the controls, a significant (p < 0.05) decrease was demonstrable only with the highest concentration evaluated (50 μg/ml). Thus, at this
concentration, even if the kinetic differences were accounted for, the HD T-LPRs were significantly depressed compared to the normal controls.

DISCUSSION

In the present study we assessed the ability of purified peripheral blood T lymphocytes from patients with HD to undergo PHA-induced mitogenesis. As a prerequisite, it was necessary to first establish the optimal dose (50 μg/ml) and time required (6 days) to induce maximal PHA-induced T-LPRs in normal subjects. When compared to normals, T cells from 12 previously untreated patients with HD (stages I–III) exhibited significant impairments of T-LPRs using both optimal and suboptimal PHA concentrations and a 6-day incubation period. When analyzed individually, 6 patients exhibited T-LPRs below the range of normals using optimal PHA concentrations, and 9 exhibited subnormal T-LPRs using suboptimal (25 μg/ml, 12.5 μg/ml) concentrations. Thus, our findings with purified T cells are similar to what has been reported using MC.22,23 That is, the defects we observed in PHA-induced T-LPRs were more apparent when suboptimal concentrations of PHA were used. In our study, 10 of 12 patients exhibited subnormal T-LPRs with at least 2 of the 3 PHA concentrations studied.

Further analysis of our findings revealed that the abnormal T-LPRs of patients with HD were characterized by kinetic differences when compared to those of normal T cells. In general, peak proliferative activity occurred earlier in the HD patients (day 4). Indeed, using suboptimal PHA concentrations, day 4 T-LPRs of the HD patients were not significantly different from the day 6 T-LPRs of controls. However, with optimal PHA concentrations, the maximal 4-day T-LPRs of the HD patients remained significantly depressed compared to the normal 6-day T-LPRs. Thus, in order to document the subtle abnormalities of T-LPRs in HD, it was necessary to perform both a dose–response curve as well as a kinetic analysis.

The depressed T-LPRs that frequently characterize patients with HD may result from several independent mechanisms, namely (1) impairment due to the presence of immunomodulating serum factors, (2) suppression mediated by non-T suppressor cells (e.g., monocytes) or by factors released by such cells (e.g., prostaglandins), (3) impairment resulting from primary T-cell defects, or from (4) immunoregulatory imbalances among the different helper/suppressor T-cell subsets. In previous studies from several laboratories it has been demonstrated that circulating suppressor monocytes,4,7 monocyte-derived inhibitory products,4 and other circulating serum inhibitory products (e.g., E-rosette inhibitory glycolipid)4,10,24 contribute to the impairment of T-LPRs. In the present studies we have focused on mechanisms 3 and 4. To accomplish this we utilized purified T cells and took several precautions to minimize any possible suppressive influence of monocytes or serum inhibitory factors (SIFs). Monocyte contamination was minimized by using a two-step purification sequence in which we depleted phagocytic cells prior to isolating the T cells by E-rosette formation. In addition, it has been demonstrated that a short-term incubation of lymphocytes with 20% FCS is sufficient for removing membrane-bound SIFs.9 Therefore, in our study, the influence of SIFs were minimized by (1) strictly avoiding contact with autologous serum, (2) incubating mononuclear cells in 20% FCS for 1 hr prior to isolating T cells, (3) including 25% FCS in the E-rosette procedure, (4) incubating T cells overnight in 20% FCS prior to assay, and (5) performing the LPR assay in 20% FCS (same lot) rather than human serum. Under the conditions we employed, all of the patients exhibited normal percentages of E-RFC. Thus, our results suggest that SIFs were not playing a major inhibitory role, and therefore, the most likely explanation for the impaired T-LPRs was an intrinsic defect of T cells or of a subpopulation of T cells. Several other possible explanations could not fully be excluded, namely, that (1) a humoral inhibitory factor remained membrane-bound after the FCS incubations, which had the capacity to interfere with T-cell mitogenesis but not with E-rosette formation, or that (2) a membrane-bound factor was shed during the PHA assay and subsequently led to impairment of T-LPRs. However, these latter possibilities would be impossible to document unless specific factors could be identified.

Recently, several functional defects of purified T cells have been described in HD. These include an impairment of locomotor activity12,13 and an impairment in proliferative capacity in autologous mixed lymphocyte reactions.25 Although our observations support the concept of a primary T-cell defect, it is also possible that the abnormalities of T-LPRs could have resulted from imbalances of immunoregulatory T-cell subset proportions rather than from an intrinsic T-cell defect. Human immunoregulatory T cells have been subclassified either by identifying specific surface Fc receptors14 or by employing hybridoma-produced monoclonal antibodies capable of distinguishing phenotypically and functionally distinct subsets of T cells.26 Although initial evidence suggested that Tγ cells contain a population of suppressor cells, whereas Tμ cells contain a population of helper cells,14 other recent investigations have not
demonstrated such distinctions. Nevertheless, since HD is associated with the presence of spontaneous suppressor T cells and with elevations in the proportions of circulating Tγ cells, it was of interest to determine whether the abnormal T-LPRs were related to an imbalance of these T-cell subsets. However, we could not identify any relationship between elevated Tγ proportions and depressed T-LPRs, thus suggesting that the depressed T-LPRs did not result from excessive suppression mediated by Tγ cells. Since the present investigation did not identify T-cell subsets by serologic methods, we cannot exclude the possibility that a relationship might exist between monoclonal antibody-defined suppressor T cells and depressed T-LPRs.

It is becoming more apparent that no single explanation can account for the full spectrum of immunodeficiencies associated with HD. Rather, the impaired in vivo expression of cell-mediated immunity characteristic of this disease probably reflects many different contributing mechanisms. The results of the present investigation and of other recent studies indicate that intrinsic functional defects of circulating T cells contribute, at least in part, to the impaired immunity in HD.

ACKNOWLEDGMENT

The authors are grateful to the physicians on the Lymphoma Service at Memorial Hospital for allowing us to study several of their patients.

REFERENCES


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