PHA Responsiveness and Subpopulations of Circulating Lymphocytes in Pernicious Anemia

By A. C. MacCuish, S. J. Urbaniak, A. H. Goldstone, and W. J. Irvine

Lymphocyte transformation responses to the mitogen phytohemagglutinin (PHA) were measured in 20 patients with proven pernicious anemia (PA) and 20 matched controls using \(^3\)H-thymidine label. The patients with PA showed significant depression of lymphocyte transformation to the three doses of PHA employed, as judged by beta counting; however, radioautographic examination of PHA-stimulated cells indicated that the results were due to a failure of intranuclear incorporation of \(^3\)H-thymidine by PA lymphocytes, rather than a failure of PHA to induce blastogenesis. The percentages and numbers of T and B lymphocytes in peripheral blood were measured in 30 patients and controls by rosette and immunofluorescence techniques, respectively. There was no significant difference in the B cell subpopulations between patients and controls; the T cell subpopulation was slightly lower in the PA patients (mean 62.4%) than in the controls (mean 65.5%), but the difference was not statistically significant. The depressed uptake of \(^3\)H-thymidine by stimulated lymphocytes in PA would seem to reflect a chemical defect rather than inherent immunologic abnormality.

Recent studies of autoimmune phenomena in pernicious anemia (PA) have examined the role of cell-mediated immune mechanisms in this disease. Using the leukocyte migration technique,\(^1\) cellular hypersensitivity has been demonstrated in PA to human intrinsic factor,\(^2,3\) hog intrinsic factor,\(^4\) human gastric mucosa,\(^5\) human gastric juice fractions,\(^6\) and liver mitochondria.\(^3,5\) Other investigators have employed antigen-induced lymphocyte transformation\(^7\) as an in vitro test to demonstrate cellular hypersensitivity against human gastric mucosa, gastric juice, and intrinsic factor in PA.\(^8\)

In the present investigation we have measured the lymphocyte transformation response to the mitogen phytohemagglutinin (PHA)\(^9\) in patients with PA and in normal subjects; in addition we have used erythrocyte rosette\(^10,11\) and indirect fluorescence\(^10,12\) techniques to compare the subpopulations of circulating T and B lymphocytes in peripheral blood from patients and controls.

PATIENTS AND METHODS

Patients

Forty patients with PA were studied. Thirty-three were women and seven men, aged from 29 to 86 yr (mean 59.8). The diagnosis of PA had been made by the usual criteria (anemia, low serum vitamin \(B\)\(_{12}\), megaloblastic bone marrow, achlorhydria, low Schilling test corrected with intrinsic factor), and all patients were receiving maintenance therapy with intramuscular vitamin \(B\)\(_{12}\). The 40 control subjects were matched for age and sex as closely as possible with the pa-
Table 1. Results of PHA Transformation Tests on Peripheral Blood Lymphocytes

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Control</th>
<th>PHA 0.3</th>
<th>PHA 0.6</th>
<th>PHA 1.2</th>
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<td>32</td>
<td>F</td>
<td>&lt;1</td>
<td>7.1</td>
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<tr>
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<td>20.2</td>
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</table>

Tests performed in 20 PA patients and 20 matched controls, using three concentrations of the mitogen. Response is measured by the uptake of $^3$H-thymidine and expressed as cpm.

Patients; most were healthy volunteers, and a few were hospital outpatients who were not known to have immunologic abnormality.

Twenty PA patients and their corresponding controls (Table 1) were studied by the PHA test. Measurement of total lymphocyte counts and T and B cell subpopulations (expressed both as percentages and absolute numbers of lymphocytes) was made in 30 PA patients and corresponding controls.

Collection of Lymphocytes

Venous blood was withdrawn from patients and controls and anticoagulated with preservative-free heparin (Evans Medical). The lymphocytes were separated from whole blood by density
PHA responsiveness in pernicious anemia

Centrifugation on a Ficoll-Triosil gradient and washed three times in Eagle's Basal Medium (EBM, Wellcome Reagents Ltd.). The cells were then resuspended in EBM with 10% fetal calf serum (Wellcome), counted, and the numbers adjusted to \( 1 \times 10^6 \) cells/ml. Cell suspensions contained more than 95% lymphocytes with a viability of greater than 98% on trypan blue exclusion.

**Lymphocyte Culture With PHA**

Stock PHA (PHA-P, Difco Labs) was diluted in EBM with 10% fetal calf serum to give three solutions containing, respectively, 0.32, 0.63 and 1.25 \( \mu l \) PHA/ml culture. Aliquots of 20 \( \mu l \) of each solution were pipetted into the wells of Cooke Microtiter trays (Flow Laboratories Ltd.) and 2 \( \times 10^5 \) cells added to each well. Triplicate cultures were performed at all concentrations of PHA in addition to a control row which contained no PHA. The microtrays were gassed with an air-5% CO₂ atmosphere and incubated at 37°C in sealed containers.

\( ^3H \)-thymidine of specific activity 5 Ci/mmole (Radiochemical Centre, Amersham) was diluted with sterile saline to give a working concentration of 2 \( \mu Ci/100 \mu l \). Twenty microliters of this solution (i.e., 0.4 \( \mu Ci \)) was added to each culture after 44 hr incubation. The cultures were regassed and incubated for a further 4 hr at 37°C. The contents of the wells were then pipetted onto glass-fiber filter papers (Whatman GF/C) which were air-dried, washed successively with cold 5% trichloroacetic acid, phosphate-buffered saline, and absolute methanol, and finally placed in Packard glass counting vials. Five milliliters scintillation fluid (NE233, Nuclear Enterprises Ltd.) was added to each vial and the samples counted for 60 sec in an automatic beta counter (Packard Tricarb 2425). Results are expressed as counts per minute (cpm).

**Lymphocyte Culture for Radioautography**

After initial results had indicated PHA response to be depressed in PA, as judged by \( ^3H \)-thymidine uptake, the phenomenon was further investigated by radioautography. For this purpose cells were prepared from four PA patients (three of whom had shown depressed PHA response) and two normal subjects. Culture conditions were as stated, but each culture was set up in aliquots of \( 1 \times 10^6 \) lymphocytes and was performed in quadruplicate. One aliquot was prepared for beta counting; the remainder were washed four times in EBM with 10% fetal calf serum, resuspended in 0.1 ml 100% fetal calf serum, and smeared onto gelatin-coated slides. The slides were air-dried, fixed, and dipped in Ilford K2 nuclear emulsion. They were then exposed in a darkroom for 24 hr at 4°C, developed in Kodak D19, and fixed in 30% sodium thiosulfate. Finally they were washed and stained with May-Grunwald-Giemsa.

The prepared slides were examined microscopically to assess the degree of morphologic blast transformation and the percentage of "labeled" blasts showing grains (i.e., indicating \( ^3H \)-thymidine uptake). Grain count analysis was further performed on labeled blasts. All slides were read without knowledge of the subject’s clinical status or PHA response.

**Identification of T Lymphocytes by Sheep Erythrocyte Rosettes**

The technique used was derived from that of Jondal et al. and incorporated the modifications of Stjernsward et al.

**Identification of B Lymphocytes**

The method used was an indirect fluorescence technique which detects surface immunoglobulins as the marker of B cells.

**RESULTS**

**Lymphocyte Transformation With PHA**

The transformation responses of lymphocytes from PA patients and controls are presented in Table 1. The mean dose–response curve to PHA in the patients is depressed by comparison with the controls (Fig. 1), and the difference between patients and controls is significant at each concentration of PHA em-

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employed ($p < 0.01$ by the Wilcoxon test). Thirteen of the patients ($65\%$) showed a dose-response curve that fell more than 1 standard deviation below the mean curve of normal subjects.

**Transformation and $^3$H-thymidine Uptake Assessed by Radioautography**

Lymphocytes from the four PA patients and two normal controls examined by this technique showed no significant differences in the degree of blast transformation to PHA (mean blast percentage $56\%$, range $46\%$-$65\%$). These figures probably underestimate total blast cell percentage as some lymphocytes are removed by the initial washing procedure. However, assessment of the percentage of grained ($^3$H-labeled) blasts showed a close relationship between the extent of labeling and the cellular radioactivity uptake as judged by beta counting (Table 2): the three subjects with high beta counts showed an average of $35\%$ labeled blasts, while the three PA patients with low beta counts showed an average of only $17\%$ labeled blasts. Analysis of the number of grains per labeled cell showed no difference between the cells of subjects with high or low beta counts.

**T and B Lymphocyte Subpopulations**

These results are presented in Table 3. The T cell counts, as judged by E-rosettes, were slightly lower in the PA patients (mean $1007$ cells/mm$^3$ ±$103$

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age/Sex</th>
<th>Blasts (%)</th>
<th>Labeled Blasts (%)</th>
<th>Beta cpm x $10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>74/F</td>
<td>54</td>
<td>46</td>
<td>304</td>
</tr>
<tr>
<td>Normal</td>
<td>37/F</td>
<td>54</td>
<td>31</td>
<td>171</td>
</tr>
<tr>
<td>PA</td>
<td>76/F</td>
<td>65</td>
<td>28</td>
<td>156</td>
</tr>
<tr>
<td>PA</td>
<td>63/F</td>
<td>46</td>
<td>17</td>
<td>98</td>
</tr>
<tr>
<td>PA</td>
<td>32/F</td>
<td>65</td>
<td>17</td>
<td>73</td>
</tr>
<tr>
<td>PA</td>
<td>76/F</td>
<td>50</td>
<td>16</td>
<td>62</td>
</tr>
</tbody>
</table>

The highest beta counts are found in subjects with the highest percentage of labeled blasts, and vice versa.

* Using $1 \times 10^6$ lymphocytes per culture.
### Table 3. Total Lymphocyte Counts and T and B Cell Subpopulations (Expressed as Percentages and Absolute Numbers)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Lymphocyte Count/mm³</th>
<th>T Lymphocytes</th>
<th>B Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent Number/mm³</td>
<td>Percent Number/mm³</td>
<td>Percent Number/mm³</td>
</tr>
<tr>
<td>Pernicious anemia</td>
<td>1558 ± 144 (n=16)</td>
<td>62.4 ± 1.5 (n=30)</td>
<td>1007 ± 103 (n=16)</td>
</tr>
<tr>
<td>Normal controls</td>
<td>1653 ± 146 (n=25)</td>
<td>65.5 ± 1.5 (n=30)</td>
<td>1086 ± 101 (n=25)</td>
</tr>
</tbody>
</table>

Results taken from 30 PA patients and matched controls. The T cell subpopulation is slightly lower in PA, but the difference does not reach statistical significance. All results are given as mean ± SEM.

SEM) than in the controls (mean 1086 ± 107). However, the difference is not statistically significant. When the T cell counts were expressed as percentages of the total lymphocyte counts, the mean figures for patients and controls were 62.4% ± 1.5% and 65.6% ± 1.5%, respectively. Again the groups do not differ significantly.

Assessment of B cell subpopulation by indirect immunofluorescence gave virtually identical results in patients and controls. The mean number and percentage of B cells in the patients was 371 ± 45 cells/mm³ and 25.5% ± 1.7%, respectively; the corresponding figures in the controls were 366 ± 66 and 23.2% ± 2.0%, respectively.

### Relationship Between PHA Response and T Cell Subpopulation

There was no close correlation between PHA response and T cell subpopulation in the ten patients and controls whose lymphocytes were examined by both methods. However the lowest T cell percentages in this subgroup were found in the four PA patients who also showed the lowest responses to PHA.

### DISCUSSION

The transformation of cultured small lymphocytes to blast cells by PHA is widely accepted as an in vitro test of cell-mediated immunologic response,7,15 and this response is most conveniently assessed by measuring the incorporation of 3H-thymidine in stimulated cells by beta counting.16 Our results show a clear-cut depression of 3H-thymidine uptake in patients with pernicious anemia, as judged by beta counting; however, the preliminary radioautographic findings would indicate that the low beta counts found in PA are not due to failure of PHA to induce blast transformation but rather are a reflection of the failure of the blast cells to take up 3H-thymidine label. It is important to consider that this may be due to an acquired chemical defect, as a recent study17 has shown markedly impaired activity of the enzyme thymidylate synthetase in patients with PA during relapse and in other diseases causing B₁₂ deficiency. However, the same study showed that enzyme levels and intranuclear incorporation of labeled thymidine were both normal in PA patients during remission,17 and all our patients were receiving adequate maintenance therapy with B₁₂. There is
thus a discrepancy between our results and those of Haurani.\textsuperscript{17} The differences may be partly explained by the small number (six) of PA patients studied and partly by the PHA assay system adopted by this investigator: Haurani utilised a prolonged (72-hr) culture period with PHA, followed by a very short (1-hr) pulse of labeled thymidine,\textsuperscript{17} in contrast to our own assay system which has been extensively investigated in order to approach optimal labeling conditions.\textsuperscript{18}

The lymphocyte subpopulation studies provide further support for the hypothesis that chemical rather than immunologic defect is responsible for a subnormal uptake of \textsuperscript{3}H-thymidine by stimulated lymphocytes in PA. We found only a small, statistically insignificant reduction in the T cell subpopulations of the patients studied, and it is therefore unlikely that T-lymphocytopenia contributed to the low beta counts that we observed.

Further studies are required to extend the above observations to larger numbers of patients with PA, to determine whether similar abnormalities are present in patients with untreated PA, and to indicate whether they are also found in allied conditions such as those forms of atrophic gastritis without malabsorption of vitamin B\textsubscript{12}. In all such studies it would be advisable to measure mitogen-induced lymphocyte transformation not only by radioisotope labeling but also by direct examination of cellular morphology and preferably by radioautography.

ACKNOWLEDGMENT

We wish to thank Dr. S. H. Davies (Royal Infirmary) and Dr. N. Allan (Western General Hospital) for allowing us to study some of their patients.

REFERENCES

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