Inhibition of *in Vitro* Lymphocyte Transformation during Chemotherapy in Man

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Summary

The *in vitro* transformation responses of lymphocytes to stimulation with phytohemagglutinin (PHA) and smallpox vaccine (vaccinia) were studied in cells from 20 patients with ocular and malignant diseases receiving chemotherapy. The transformation of lymphocytes to lymphoblast-like cells was reduced from 71% in the pretreatment PHA-stimulated cultures to 1.5% during therapy. The response to vaccinia was reduced from 12% before therapy to 0% during therapy. The mitotic indices fell from 1.5% (PHA) and 1.2% (vaccinia) to 0% for each during therapy.

Intensive combination therapy with parenteral 6-mercaptopurine and methotrexate, with or without prednisolone completely abolished transformation after 3 days of treatment. Substantial recovery occurred within 3 days after the end of therapy. Nontoxic therapy with methotrexate or 6-mercaptopurine which did not induce leukopenia took 2–5 weeks to cause maximum suppression.

The abnormality seemed due to intrinsic damage to the lymphocytes and not to persistent antimetabolite in the plasma.

*In vitro* lymphocyte transformation is an easy and reproducible way of evaluating the immune competence of an individual's circulating lymphocytes.

Introduction

Impaired immunologic competence has been recognized in a variety of human diseases (27), and suppression of immunologic competence has been observed during certain types of therapy (16). Clinically, immunosuppressive antimetabolite therapy is used to maintain homografts (21) and in the treatment of certain "auto-immune" disorders (33). In general, antimetabolite therapy can delay homograft rejection, prevent the development of delayed hypersensitivity, inhibit the mononuclear cell phase of the local inflammatory response, and block primary antibody responses (16). Partial suppression of the secondary response has also been achieved with antimetabolites (18, 30).

Immunologically competent small lymphocytes play a central role in mammalian host defense mechanisms (8). The recently developed technics of short-term *in vitro* lymphocyte culture offer means of directly assessing the competence of an individual's peripheral blood lymphocytes. When PHA, or antigens with which the subject has had prior contact, or homologous or heterologous tissue antigens are added to cultured peripheral blood leukocytes, a proportion of the lymphocytes transform into lymphoblast-like cells and undergo mitosis (29). There is no detectable response to primary antigens. The transformed lymphocytes are morphologically similar to the pyroninophilic lymph node cells which develop in nodes draining sites of recent antigenic stimulation (1, 34).

Impaired *in vitro* lymphocyte transformation has been noted in several disorders where there is impaired immunologic competence. These include chronic lymphocytic leukemia (25), Hodgkin's disease (12), ataxia telangiectasia (23), and Boeck's sarcoid (15). The above mentioned observations suggest that the *in vitro* lymphocyte transformation response reflects the immunologic competence of an individual's circulating lymphocytes.

In the current study *in vitro* leukocyte cultures with PHA and smallpox vaccine (vaccinia) were used to evaluate the immunologic competence of lymphocytes from patients receiving chemotherapy. Suppression of *in vitro* lymphocyte transformation was noted. This was felt to reflect the effect the therapy would have on secondary immune responses and therefore its potential effect on homograft rejection, "auto-immune" disorders, and resistance to infection.

Materials and Methods

Lymphocyte Culture Technic

Fifty ml of venous blood were mixed with 500 units of heparin (heparin sodium, Upjohn) and allowed to sediment at 37°C for 2 hr in 150- x 20-mm screw-cap tubes. The supernatant WBC-rich plasma obtained had a count of 1000–4000 WBC/cm3 with approximately 50% lymphocytes (range: 30–70%).

WBC-rich plasma was added to Eagle's minimal essential medium in a ratio of 1:2 and divided into 6- or 12-ml aliquots for culture. The medium was supplemented with 100 units of penicillin, 100 units of streptomycin, and 50 μg of glutamine per ml (all from Flow Labs, Rockville, Md.).

Each set of cultures consisted of an unstimulated control culture, a culture containing 0.2 ml of phytohemagglutinin-M (Difco Labs, Detroit, Mich.), and 3 cultures containing 0.5 ml of 1:10, 1:100, or 1:1000 dilution of vaccinia (Dryvax-Wyeth).

1 Present in part at the Annual Meeting of the American Association for Cancer Research, held at Philadelphia, Pa., April 9, 1965.

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Inhibition of Lymphocyte Transformation

### TABLE 1

<table>
<thead>
<tr>
<th>GROUP No.</th>
<th>No. of patients</th>
<th>DIAGNOSIS</th>
<th>THERAPY</th>
<th>Drug</th>
<th>Dose</th>
<th>Route</th>
<th>Schedule</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>Acute leukemia</td>
<td></td>
<td>6-MP</td>
<td>500 mg/sq m</td>
<td>i.v.</td>
<td>Daily</td>
<td>5 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MTX</td>
<td>7.5 mg/sq m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prednisolone</td>
<td>1000 mg/sq m</td>
<td>i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Acute leukemia</td>
<td></td>
<td>6-MP</td>
<td>1000 mg/sq m</td>
<td>i.v.</td>
<td>Daily</td>
<td>7 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MTX</td>
<td>15 mg/sq m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Prednisolone</td>
<td>1000 mg/sq m</td>
<td>i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>Acute leukemia</td>
<td></td>
<td>6-MP</td>
<td>500 mg/sq m</td>
<td>i.v.</td>
<td>Daily</td>
<td>5 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MTX</td>
<td>7.5 mg/sq m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Uveitis</td>
<td></td>
<td>MTX</td>
<td>25 mg/sq m</td>
<td>i.v.</td>
<td>Q4d</td>
<td>42 days</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>Carcinoma</td>
<td></td>
<td>5-FU</td>
<td>7.5 mg/kg</td>
<td>i.v.</td>
<td>Daily</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MTX</td>
<td>0.75 mg/kg</td>
<td>i.v.</td>
<td>Q2d</td>
<td>5 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cyclophosphamide</td>
<td>7.5 mg/kg</td>
<td>i.v.</td>
<td>Q2d</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vincristine</td>
<td>0.025 mg/kg</td>
<td>i.v.</td>
<td>Weekly</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Hemolytic anemia</td>
<td></td>
<td>6-MP</td>
<td>25-50 mg/sq m</td>
<td>i.v.</td>
<td>Daily</td>
<td>30 days</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>Acute leukemia</td>
<td></td>
<td>Cytosine arabinoside</td>
<td>25-50 mg/sq m</td>
<td>i.v.</td>
<td>Daily</td>
<td>12 weeks</td>
</tr>
</tbody>
</table>

* 6-MP, 6-mercaptopurine; MTX, methotrexate; 5-FU, 5-fluorouracil; cytosine arabinoside, 1-β-D-arabinofuranosylcytosine; Q4d, every 4th day; Q2d, every other day.

respectively. Cultures were incubated for 5 days at 37°C in stationary stoppered bottles in air.

### Harvesting and Counting

Polystyrene particles (0.1 ml, 1:100 saline dilution, 1.3 μm diameter; Dow Chemical Co., Midland, Mich.) and 4 mg of colcemide (Ciba Pharmaceutical, Summit, N. J.) were added to each culture 4 hr before harvesting. The polystyrene particles are usually phagocytized by macrophages and other phagocytic cells, thus aiding in their differentiation from the lymphoblast-like transformed cells (28). Colcemide arrests mitosis in metaphase and thus makes the mitotic index of the cultures easier to count (22).

The cultured cells were collected by centrifugation at 1400 rpm for 7 min (International Centrifuge, No. 240 head), fixed in a 1:9 glacial acetic acid:ethyl alcohol mixture for 10 min, re-centrifuged, resuspended in fixative, pipetted on slides, air dried, and stained with Giemsa (Harleco, A. H. Thomas, Philadelphia Pa.).

### TABLE 2

<table>
<thead>
<tr>
<th>GROUP No.</th>
<th>Control</th>
<th>Therapy</th>
<th>PHA Transformation response (%)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blasts</td>
<td>Mitoses</td>
<td>Blasts</td>
<td>Mitoses</td>
</tr>
<tr>
<td>1</td>
<td>73</td>
<td>3.0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>2.6</td>
<td>7</td>
<td>0</td>
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<tr>
<td>4</td>
<td>72</td>
<td>2.0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>4.0</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>3.0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>81</td>
<td>5.0</td>
<td>46</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a The groups are the same as in Table 1. Values given are means.
a PHA, phytohemagglutinin.
* Discharged at termination of therapy.
* Started on another drug immediately after cessation of cytosine arabinoside.
Evan M. Hersh and Joost J. Oppenheim

Five hundred cell differential counts and 1000 cell mitotic indices were done on each side. Cells counted included transformed lymphocytes, small untransformed lymphocytes, macrophages, dead cells, polymorphonuclear leukocytes, and mitoses.

Transformed lymphocytes were characterized by their large size, a large oval nucleus, 1–4 prominent nucleoli, heterochromatic nucleoplasm and basophilic nongranular cytoplasm. Small untransformed lymphocytes were small in size, had clumped nuclear chromatin, no nucleoli, and scanty pale cytoplasm. Macrophages were large cells, had intermediate-sized nuclei, often with light staining nucleoli, and abundant granular cytoplasm filled with phagocytized polystyrene particles. Their cytoplasm:nucleus ratio was estimated at about 2. Dead cells were characterized by disruption of the nuclear membrane and/or cytoplasmic membranes and often showed karyolysis, karyorrhexis, or pyknosis of the nucleus.

Patients Studied and Therapy Administered

Two hundred fifteen sets of peripheral blood leukocyte cultures were done before, during, and after chemotherapy in 20 patients, (Table 1). The patients with acute leukemia were all in peripheral blood remission (no circulating leukemic cells) when studied. Seven patients with acute leukemia were given intensive combination chemotherapy with 5-day courses of i.v. 6-MP, MTX, and prednisolone. Two patients with acute leukemia received more intensive 7-day courses of the same drugs. Two patients with acute leukemia received 5-day courses of i.v. 6-MP and MTX without prednisolone. Intermittent i.v. MTX therapy was given to 4 patients who were in good health except for uveitis. Three patients with metastatic carcinomas received combination therapy with 5-fluorouracil, MTX, cyclophosphamide, and vincristine in 5-day courses. Finally, 1 patient with hemolytic anemia receiving daily p.o. 6-MP and 1 with acute leukemia receiving cytosine arabinoside were also studied. Patients receiving intensive 5-day therapy (Groups 1, 3, and 5) were tested only twice during and 2–4 times after each particular course of therapy. Samples were obtained on different days in different patients so that values were available for each day during and 5 days after therapy. Patients in Groups 2, 4, 6, and 7 were tested weekly. Each patient served as his own control while not on therapy. Control values were obtained before any therapy except in the patients with acute leukemia, where control values were obtained between courses.

Results

The Control Transformation Response

The control responses to PHA and vaccinia in the 20 patients are seen in Chart 1. After 5 days of culture with PHA, 71% of the cells in the cultures were transformed lymphocytes or lymphoblasts; 1.5% of these cells were in mitosis. With vaccinia, 12% of the cells were transformed cells and 1.2% were in mitosis. The responses to PHA were identical with the responses of the 16 normal individuals studied and are similar to the observa-
Inhibition of Lymphocyte Transformation

Chart 1 also shows the over-all effects of therapy. Each type of therapy resulted in an inhibition of lymphocyte transformation and a fall in the mitotic index from control levels. This occurred to a similar degree and at a similar rate in both the PHA- and the vaccinia-stimulated cultures. The maximally inhibited cultures showed no transformed cells but only small lymphocytes and dead cells. Even the response of the patient with the low control value fell further during therapy (34–0% in response to PHA). In the pretreatment cultures most of the transformed cells were of fairly uniform size. In the cultures done during therapy, when inhibition was incomplete, those cells that did transform appeared smaller.

There were striking differences among the various types of therapy with regard to the rate of inhibition of lymphocyte transformation. The effects of the individual types of therapy are seen in Table 2. Intensive combination therapy with 6-MP and MTX (Groups 1–3) inhibited transformation rapidly and completely (Chart 2). The effect was the same whether or not prednisolone was used in conjunction with the other 2 drugs. The effects of 4-drug therapy given to the patients with metastatic carcinoma were similar (Group 5, Chart 3). The lymphocytes recovered rapidly from the inhibition. Within a median of 3 days after the end of combination 6-MP and MTX, transformation with PHA was within normal limits. However,

![Chart 3](image)

**Chart 3.** The effect of combination chemotherapy on lymphocyte transformation. The effect on lymphocyte transformation of 4-drug combination therapy in a patient with metastatic carcinoma is shown. This patient never responded well to vaccinia.

![Chart 4](image)

**Chart 4.** The effect of intensive combination therapy on lymphocyte transformation. The prolonged inhibition of lymphocyte transformation induced by intensive combination therapy given for 7 days. Note that the absolute lymphocyte count did not correlate with the transformation response and that it had risen above pretreatment levels by Day 58 while normal lymphocyte transformation did not return until Day 72. For absolute lymphocyte count each division equals 500 lymphocytes/cu mm.

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if the drugs were given at double the usual dose for 7 rather than 5 days (Group 2, Chart 4), recovery was prolonged to 25 days. In patients receiving only intermittent i.v. MTX there was a gradual fall in the percentage transformation (Chart 5). The decline did not occur until after 2-3 weeks of therapy and reached a maximum only after 5 weeks. At that time the degree of suppression was the same as for the more intensive therapy. Recovery was not studied in this group or Group 6 because the patients were discharged within a day or 2 after the end of therapy. Daily p.o. 6-MP also produced gradual, rather than rapid, suppression. Little effect was noted in the patient receiving cytosine arabinoside in spite of the fact that the patient was followed through two 6-week courses of therapy, both of which produced significant lymphopenia.

There was a clear correlation between the degree of cell death in the patients' unstimulated cultures while on therapy and inhibition of transformation in the patients' PHA-stimulated cultures (Table 3). Thus, in those sets where the unstimulated culture contained more than 50% dead cells, there was significantly less transformation in the corresponding PHA-stimulated cultures than when there were less than 50% dead cells ($P < 0.02$). This observation suggested that cell death alone might account for the impaired transformation and that the surviving lymphocytes could transform normally. To investigate this possibility, the ratio of transformed cells to untransformed lymphocytes in the patients' PHA cultures was studied (Chart 6). The majority of morphologically intact lymphoid cells in cultures done during therapy were untransformed, and therefore these cultures showed a median ratio of 0. The majority of the lymphoid cells in cultures done during the pretreatment period or after recovery were transformed and showed a median ratio of 9.5. This suggests intrinsic damage to the morphologically intact lymphocytes.

Patients' cells collected during treatment on 19 occasions were washed and resuspended in calf serum rather than in autologous plasma. Cultures were then set up in the usual way. The lymphocyte transformation response was not restored by removing autologous plasma from the patients' cells. The patients' plasma did not inhibit transformation of cells obtained from normal people.

Lymphopenia developed during the more intensive types of therapy. It did not develop, however, in the patients receiving intermittent i.v. MTX. There was a correlation between the depressed circulating lymphocyte count and the subsequent degree of inhibition of in vitro transformation observed only at levels below 500/cu mm. For circulating lymphocyte counts ranging from 500 to over 3500/cu mm (Table 4), there was no

---

**Table 3**

<table>
<thead>
<tr>
<th>No. of culture sets</th>
<th>% Transformation</th>
<th>% dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>6</td>
<td>67</td>
<td>1-80</td>
</tr>
<tr>
<td>26</td>
<td>61</td>
<td>7-80</td>
</tr>
<tr>
<td>42</td>
<td>62</td>
<td>2-80</td>
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<tr>
<td>42</td>
<td>51</td>
<td>0-82</td>
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<tr>
<td>33</td>
<td>54</td>
<td>4-80</td>
</tr>
<tr>
<td>22</td>
<td>24</td>
<td>0-72</td>
</tr>
<tr>
<td>16</td>
<td>31</td>
<td>1-68</td>
</tr>
<tr>
<td>28</td>
<td>2</td>
<td>0-45</td>
</tr>
</tbody>
</table>

* This tabulation includes sets of cultures done before, during, and after therapy. Cultures done while the patients were off therapy and responding normally to PHA always had less than 50% dead cells.
The Effect of Chemotherapy on In Vitro Lymphocyte Transformation

**Discussion**

In vitro lymphocyte transformation is related to in vivo immunologic responsiveness. The transformed lymphocytes are similar morphologically to the pyroninophilic lymph node cells which develop after antigenic stimulation in vivo and which go on to form plasma cells (1, 34). In vitro transformation responses of lymphocytes cultured with antigens represent secondary responses of these lymphocytes. Thus, only subjects with prior exposure to tuberculosis (as evidenced by a positive tuberculin skin test) will show an in vitro lymphocyte transformation response to purified protein derivative (26). The subject must have had prior immunizing contact with the antigen before his lymphocytes will respond to it in vitro (6, 17). In cultures of mixed human peripheral blood lymphocytes the degree of transformation response is greater in unrelated than in related subjects and is almost completely absent in mixes of cells from monozygotic twins (3). During skin graft rejection there is a marked increase of the in vitro lymphocyte transformation response of recipient cells in mixed donor-recipient leukocyte cultures (24). A number of human diseases where abnormalities of the lymphoid tissue, impaired immunologic competence, and impaired in vitro lymphocyte transformation coexist have already been mentioned. The technic of lymphocyte culture with various mitogenic agents can be used therefore to evaluate lymphocyte competence during chemotherapy directed at inhibition of the immune response. One such situation would be to follow the effects of continuing chemotherapy on the lymphocytes of patients who have received homografts (31).

The current study has shown that several types of chemotherapy can inhibit in vitro lymphocyte transformation in man. The duration of therapy before maximum inhibition was directly related to drug dose as was the time it took for normal lymphocyte transformation to return after the end of treatment. Single-drug non-leukopenia-producing therapy inhibited transformation to the same degree as intensive combination therapy but only after a longer period of treatment. The rapid inhibition of transformation and the rapid recovery after the end of therapy are noteworthy. The degree of inhibition did not correlate with

---

**TABLE 4**

The Relationship between Circulating Lymphocyte Levels and the Lymphocyte Transformation Response

<table>
<thead>
<tr>
<th>No. of cultures</th>
<th>Absolute lymphocyte count (cells/cu mm)</th>
<th>Median transformation response to PHA* (%)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>0-500</td>
<td>3</td>
<td>0-80</td>
</tr>
<tr>
<td>54</td>
<td>500-1000</td>
<td>59</td>
<td>0-77</td>
</tr>
<tr>
<td>42</td>
<td>1000-1500</td>
<td>54</td>
<td>0-80</td>
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<tr>
<td>32</td>
<td>1500-2000</td>
<td>54</td>
<td>0-77</td>
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<tr>
<td>13</td>
<td>2000-2500</td>
<td>49</td>
<td>0-77</td>
</tr>
<tr>
<td>24</td>
<td>2500-3000</td>
<td>58</td>
<td>5-80</td>
</tr>
<tr>
<td>13</td>
<td>over 3000</td>
<td>71</td>
<td>10-80</td>
</tr>
</tbody>
</table>

* Only the group with 0-500 lymphocytes/cu mm in peripheral blood was significantly different from the other groups (P < 0.01 by chi square test).

b PHA, phytohemagglutinin.
the patients' absolute lymphocyte count except at lymphocyte levels below 500/eu mm.

Impaired in vitro transformation of lymphocytes from patients receiving chemotherapy is probably due to inhibition of the nucleic acid and protein synthesis which is a necessary part of the transformation process (7, 9, 14, 20). Although steroids can block lymphocyte transformation when added in vitro (5), steroid therapy does not explain our observations since only 9/20 patients received prednisolone.

This study outlines another type of host responsiveness which can be modified by chemotherapy in man. The suppression of in vitro lymphocyte transformation appears at approximately the same time after initiation of intensive therapy as the inhibition of the inflammatory reaction and the primary antibody response (11, 13, 32). There is complete inhibition of the primary antibody response to antigens given 24 hr after the start of intensive chemotherapy. There is a good antibody response, however, to another antigen given 24 hr after the end of the course of treatment (10). This is analogous to the rapid inhibition and recovery of transformation demonstrated in this study during and after short-term intensive therapy. Similarly, the effect of chemotherapy on transformation of peripheral blood lymphocytes can be compared to the inhibition by chemotherapy of the appearance of pyroninophilic lymph node cells after homografting or antigenic stimulation in vivo (2, 34). However, these studies were done during primary stimulation, and the type of therapy used did not block the lymph node response to secondary stimulation (2). The secondary stimulus was probably given too early in the course of therapy.

The results of the current study suggest that even therapy which does not induce leukopenia (such as intermittent MTX) would eventually suppress the secondary response. However, the most rapid and complete inhibition of immune responses, including the primary antibody response, the local inflammatory response, and in vitro lymphocyte transformation, is achieved with intensive combination 6-MP and MTX therapy (11, 13, 32). If continued immunosuppression is the objective of therapy, it might be achieved with relatively nontoxic doses of either drug.

It is noteworthy that patients who have acute leukemia but who are in remission (or at least without any circulating leukemic cells) and off therapy have normal in vitro lymphocyte transformation. Similarly, such patients have normal local inflammatory (4) and primary antibody responses (19).

Acknowledgments

We would like to thank Mrs. Emil J. Freireich and Emil Frei, III, for their guidance and support in all aspects of this work. We would also like to thank Mrs. Ligita Novikovs and Mr. Robert Colligan for their capable technical assistance.

References


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