Inhibition of in Vitro Lymphocyte Transformation during Chemotherapy in Man

EVAN M. HERSH* AND JOOST J. OPPENHEIM
Medicine Branch, National Cancer Institute, NIH, Bethesda, Maryland

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, “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/cu mm 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 /ig 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),...
Inhibition of Lymphocyte Transformation

TABLE 1
THE PATIENTS’ DIAGNOSES AND THERAPY RECEIVED

<table>
<thead>
<tr>
<th>GROUP No.</th>
<th>No. of patients</th>
<th>Diagnosis</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>Acute leukemia</td>
<td>6-MP*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTX</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prednisolone</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Acute leukemia</td>
<td>6-MP*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTX</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prednisolone</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>Acute leukemia</td>
<td>6-MP*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTX</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Uveitis</td>
<td>MTX</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>Carcinoma</td>
<td>5-FU</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vincristine</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Hemolytic anemia</td>
<td>6-MP*</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>Acute leukemia</td>
<td>Cytosine arabinoside</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Route</th>
<th>Schedule</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-MP</td>
<td>500 mg/sq m</td>
<td>i.v.</td>
<td>Daily</td>
<td>5 days</td>
</tr>
<tr>
<td>MTX</td>
<td>7.5 mg/sq m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>1000 mg/sq m</td>
<td>i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>1000 mg/sq m</td>
<td>i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>15 mg/sq m</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>1000 mg/sq m</td>
<td>i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>500 mg/sq m</td>
<td>i.v.</td>
<td>Q4d</td>
<td>42 days</td>
</tr>
<tr>
<td>MTX</td>
<td>7.5 mg/sq m</td>
<td>i.v.</td>
<td>Q2d</td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>25 mg/sq m</td>
<td>i.v.</td>
<td>Q2d</td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>2.0 mg/sq m</td>
<td>p.o.</td>
<td>Daily</td>
<td>30 days</td>
</tr>
<tr>
<td>MTX</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-beta-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 μ diameter; Dow Chemical Co., Midland, Mich.) and 4 μg 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; recentrifuged, resuspended in fixative, pipetted on slides, air dried, and stained with Giemsa (Harleco, A. H. Thomas, Philadelphia Pa.).

TABLE 2
THE EFFECT OF CHEMOTHERAPY ON IN VITRO LYMPHOCYTE TRANSFORMATION

<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>
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<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>75</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>

* The groups are the same as in Table 1. Values given are means.

PHA, phytohemagglutinin.

Discharged at termination of therapy.

Started on another drug immediately after cessation of cytosine arabinoside.
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. The response to vaccinia was more variable than to PHA. The rapidity of inhibition and recovery is noteworthy.
Inhibition of Lymphocyte Transformation

The Effects of Therapy

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,
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 re-
ceiving 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 signifi-
cantly 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 there-
fore 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 morphologi-
cally 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 lympho-
cyte 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>
<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>
</tr>
<tr>
<td>42</td>
<td>51</td>
<td>0-82</td>
</tr>
<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.
Inhibition of Lymphocyte Transformation

THE EFFECT OF CHEMOTHERAPY ON IN VITRO LYMPHOCYTE TRANSFORMATION

TABLE 4

<table>
<thead>
<tr>
<th>No. of cultures</th>
<th>Absolute lymphocyte count (cells/cu mm)</th>
<th>Median transformation response to PHAa (%)</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>
</tr>
<tr>
<td>32</td>
<td>1500-2000</td>
<td>54</td>
<td>0-77</td>
</tr>
<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>

a 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.

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 technique 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

Correlation. The patients with lymphocyte counts below 500/cu mm were all under intensive therapy when their counts reached a low point. In some patients the transformation response returned to normal prior to the resolution of the lymphopenia or even while the lymphopenia was worsening. In others the lymphocyte count returned to normal before the lymphocyte transformation response recovered (Chart 4).

Cells were added to the cultures in final concentrations ranging from 350 to 1300 WBC/cu mm. Within these limits the cell concentration in the cultures did not correlate with the PHA transformation response.
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 leukaemia (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 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.

Acknowledgments

We would like to thank Drs. 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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