Augmentation of the Human Immune Response by Cyclophosphamide

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ABSTRACT

Pretreatment of experimental animals with cyclophosphamide (CY) markedly potentiates the acquisition of T-cell-mediated immunity to an antigen given 1 to 4 days later. We have examined the primary induction of delayed-type hypersensitivity (DTH) and antibody to keyhole limpet hemocyanin (KLH) and DTH to 1-chloro-2,4-dinitrobenzene (DNCB) in 22 patients receiving CY for metastatic cancer, 12 with melanoma and 10 with colorectal carcinoma. Sixteen days before CY, one-half of the patients received KLH and one-half received DNCB; 3 days after CY, they received KLH or DNCB, whichever they had not received initially. Blood was drawn for antibody titer and/or skin testing was performed 14 days after administration of antigen. For each antigen, the responses of pre-CY patients were compared with those of post-CY patients.

We found that pretreatment of patients with CY significantly augmented the development of DTH to KLH. The DTH reactions of the group of patients given KLH 3 days after CY were significantly greater than those of the group of patients given KLH without CY (medians: KLH alone, 0; KLH after CY, 18 mm; p = 0.025). With CY pretreatment, 11 of 11 patients developed a DTH response of greater than 5 mm compared with 4 of 11 patients without CY (p = 0.002). No patient developed DTH to DNCB when it was given without CY whereas 3 of 11 patients developed DTH when DNCB was given 3 days after CY (p = 0.082). CY pretreatment neither augmented nor suppressed the antibody response to KLH; the proportion of patients with antibody 14 days after antigen was 2 of 11 without CY and 4 of 11 with CY pretreatment (p = 0.24).

It appears that CY pretreatment resulted in the development of DTH responses in otherwise unreactive patients. Reversal of the T-cell anergy of advanced cancer could lead to augmentation of the immune response to tumor-associated antigens.

INTRODUCTION

CY was developed as an antitumor drug by Brock and Wilmanns (4) and has become one of the most widely used agents in the chemotherapy of human cancer. Early studies with CY demonstrated that it was a potent immunosuppressive in experimental animals (15) and in humans (19). However, during studies of the effect of CY on allergic contact dermatitis in guinea pigs, Maguire and Ettore (14) came upon the surpris-
 augmentation of immunity by cy

Table 1
Summary of clinical characteristics of patients

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of melanomas/no. of colon cancer</th>
<th>Median age (yr)</th>
<th>Sex</th>
<th>Median Karnovsky status</th>
<th>No. of prior chemotherapies</th>
<th>Nodes and skin only</th>
<th>Visceral</th>
<th>No. of lymphocytes/ cu mm blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6/5</td>
<td>59 (39-69)</td>
<td>8/11 men</td>
<td>70 (30-90)</td>
<td>3 (2-4)</td>
<td>2</td>
<td>9</td>
<td>895 ± 260</td>
</tr>
<tr>
<td>II</td>
<td>6/5</td>
<td>62 (36-71)</td>
<td>3/11 men</td>
<td>70 (30-80)</td>
<td>3 (2-5)</td>
<td>2</td>
<td>9</td>
<td>990 ± 145</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, range.

† Mean ± S.E.; significantly lower than controls (1838 ± 134) by t test (p < 0.01).

‡ Group I versus Group II, p < 0.05, Fisher's exact test.

Table 2
Detailed listing of clinical characteristics of patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Karnovsky status</th>
<th>Metastatic sites</th>
<th>Prior chemotherapy</th>
<th>Survival (mos.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A. T.</td>
<td>62</td>
<td>M</td>
<td>80</td>
<td>Lu</td>
<td>DT, DB, meCN, T</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>J. O.</td>
<td>39</td>
<td>M</td>
<td>40</td>
<td>s.c., Bo</td>
<td>DT, BN, DB</td>
<td>1</td>
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<tr>
<td></td>
<td>R. C.</td>
<td>68</td>
<td>M</td>
<td>80</td>
<td>No</td>
<td>DT, BCG, meCN, DB, T</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>B. M.</td>
<td>45</td>
<td>F</td>
<td>70</td>
<td>No, Ab</td>
<td>DT, BCG, CN, V, DB</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>J. S.</td>
<td>61</td>
<td>F</td>
<td>50</td>
<td>Lu, Bo</td>
<td>Te, MG</td>
<td>5</td>
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<tr>
<td></td>
<td>W. B.</td>
<td>59</td>
<td>M</td>
<td>70</td>
<td>Lu, Li</td>
<td>FU, MU, MG</td>
<td>6</td>
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<tr>
<td></td>
<td>J. B.</td>
<td>69</td>
<td>M</td>
<td>70</td>
<td>Lu</td>
<td>DT, meCN, DB, Ta</td>
<td>7</td>
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<tr>
<td></td>
<td>H. C.</td>
<td>52</td>
<td>M</td>
<td>30</td>
<td>Ab</td>
<td>FU, MU</td>
<td>1</td>
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<tr>
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<td>L. C.</td>
<td>59</td>
<td>M</td>
<td>40</td>
<td>Li</td>
<td>FU, HT, DT, Mi</td>
<td>3</td>
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<tr>
<td></td>
<td>J. B.</td>
<td>58</td>
<td>M</td>
<td>70</td>
<td>Lu</td>
<td>FU, MG</td>
<td>10+</td>
</tr>
<tr>
<td></td>
<td>B. S.</td>
<td>49</td>
<td>F</td>
<td>90</td>
<td>s.c.</td>
<td>BCG, meCN, DT</td>
<td>6+</td>
</tr>
<tr>
<td>II</td>
<td>S. P.</td>
<td>56</td>
<td>M</td>
<td>80</td>
<td>Lu</td>
<td>meCN, DB, DT</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>B. P.</td>
<td>66</td>
<td>M</td>
<td>50</td>
<td>s.c., Li</td>
<td>CN, DT, DB, T, M</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>H. M.</td>
<td>61</td>
<td>F</td>
<td>70</td>
<td>s.c.</td>
<td>DB, meCN</td>
<td>26+</td>
</tr>
<tr>
<td></td>
<td>A. B.</td>
<td>70</td>
<td>F</td>
<td>80</td>
<td>Lu, No</td>
<td>DT, meCN, DB</td>
<td>19+</td>
</tr>
<tr>
<td></td>
<td>S. A.</td>
<td>36</td>
<td>F</td>
<td>80</td>
<td>s.c.</td>
<td>DT, DN, DB</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>M. T.</td>
<td>59</td>
<td>F</td>
<td>70</td>
<td>Lu</td>
<td>FU, CN</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>J. C.</td>
<td>66</td>
<td>M</td>
<td>60</td>
<td>Lu</td>
<td>FU, DB, meCN, DT, Ch</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>S. M.</td>
<td>56</td>
<td>F</td>
<td>90</td>
<td>Li</td>
<td>FU, MG, Mi, DT</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>D. W.</td>
<td>71</td>
<td>M</td>
<td>50</td>
<td>No, Lu</td>
<td>DB, meCN, DT</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>J. M.</td>
<td>70</td>
<td>F</td>
<td>30</td>
<td>Ab</td>
<td>FU, Mi, DT</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>F. D.</td>
<td>62</td>
<td>F</td>
<td>80</td>
<td>Li, Lu</td>
<td>FU, MG</td>
<td>6+</td>
</tr>
</tbody>
</table>

* Lu, lung; DT, dithiосiocyanate (dacarbazine); DB, dibromomodulcitol (NSC 104800); meCN, methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (semustine; NSC 95441); T, trimethyl colchicinic acid (NSC 36354); Bo, bone; BN, 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine); No, lymph nodes; BCG, Bacillus Calmette-Guérin; Ab, diffuse abdominal; CN, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); V, vincristine; Te, tegafur (NSC 145950); MG, methylglyoxal bis(guanylhydrazine) (NSC 32946); Li, liver; FU, fluorouracil; HU, hydroxyurea; Ta, tamoxifen; Mi, mitomycin; M, methotrexate; Ch, chlorozotocin (NSC 178248).

Antigens. KLH was obtained from Calbiochem-Behring Corp. (San Diego, Calif.) and purified by the method of Campbell et al. (5). To ensure that the KLH was in the associated form, it was stored at pH 6.4, and fresh batches were prepared every 2 months. Sensitization was accomplished by injecting 1.0 mg s.c., and challenge consisted of the i.d. injection of 0.1 mg. In addition, all patients were given a separate i.d. injection of 0.1 mg KLH at the time of sensitization to document that they did not have a fortuitously established sensitivity. DTH was determined by measuring the largest and right-angle diameters of the area of induration and calculating the mean.

Antibody to KLH was measured by a passive hemagglutination assay that has been described in detail previously (2). In brief, glutationaldehyde-fixed sheep erythrocytes were treated with tannic acid, washed, and then coated with KLH. The cells were washed to remove free KLH, suspended at a 1.0% concentration in buffered saline (NaCl, 7.2 g; Na2HPO4-12 H2O, 3.6 g; KH2PO4, 0.4 g; H2O, 1000 ml) with 0.5% rabbit serum as a stabilizer, and added to round-bottomed microtiter plates (Linbro Division, Flow Laboratories, McLean, Va.) containing serial dilutions of the sera to be tested. Agglutination patterns were evaluated after 18 hr of incubation at room temperature. The antibody titer was defined as log2 of the dilution of serum that produced any observable degree of agglutination compared to that seen with diluent alone. An aliquot of each serum was treated with 2-mercaptoethanol before testing to inactivate IgM.

DNCB was obtained from BDH Chemicals (Poole, England).
and was freshly prepared before each application by dissolving it in acetone:corn oil (9:1). Sensitization was accomplished by the topical application of 2.0 mg DNCB to a skin site on the volar surface of the forearm within the confines of a 1-cm-diameter steel ring. All patients developed a primary irritant response, and none developed a 48-hr DTH response. Challenge consisted of the topical application of 4 concentrations of DNCB (0.050, 0.025, 0.013, and 0.006 mg/0.1 ml) to separate skin sites on the forearm. DTH reactions were scored as positive if any of the concentrations produced a full circle of erythema and induration after 48 hr.

CY was administered in a dose of 1000 mg/sq m by rapid i.v. injection. All patients received prochlorperazine to ameliorate nausea and vomiting.

RESULTS

KLH DTH. The most striking finding of this study was that pretreatment of patients with CY significantly augmented the development of DTH to KLH (Chart 2). The DTH reactions of patients given KLH 3 days after CY (Group II) were significantly greater than those of patients given KLH without prior CY (Group I) (medians: KLH alone, 0; KLH after CY, 18 mm; p = 0.025, Mann-Whitney U test). With CY pretreatment, 11 of 11 patients developed a DTH response of greater than 5.0 mm compared to 4 of 11 without CY (p = 0.002, t test for proportions).

Although there was a difference in the sex distribution of the 2 groups, within each group the proportion of men and women with a positive DTH response was nearly identical (number with DTH >5.0 mm: Group I men, 3 of 8; Group I women, 1 of 3; Group II men, 3 of 3; Group II women, 8 of 8).

DNCB DTH. Of 10 patients given DNCB without CY, none developed DTH. (One patient was not pretested.) Of 11 patients given DNCB 3 days after CY, 3 developed a positive DTH response. The difference (0 of 10 versus 3 of 11) was of borderline significance (p = 0.082, t test for proportions). The lowest challenge doses of DNCB that induced DTH in the 3 responding patients were 0.025, 0.013, and 0.006 mg, respectively.

KLH Antibody Response. As shown in Chart 3, pretreatment with CY neither augmented nor suppressed the agglutinating antibody response to KLH. The proportion of patients who had detectable antibody 14 days after antigen was 2 of 11 without CY and 4 of 11 with CY pretreatment (p = 0.24, t test for proportions). In both groups, the 14-day antibody was predominantly mercaptoethanol sensitive (i.e., IgM). Three patients from whom follow-up sera were obtained showed a shift to mercaptoethanol-resistant (i.e., IgG) antibody. There was no correlation, either positive or negative, between DTH and antibody responses; of the patients who developed antibody, 2 developed DTH and 4 did not.

Response to CY and Survival. Three of the 22 patients (13.6%) demonstrated a partial regression of tumor following CY administration. Two of these were melanoma patients who had a greater than 50% shrinkage of cancerous lymph nodes. The other was a patient with colon carcinoma who had a 50% decrease in the size of the liver. One of these patients was in Group I and the others were in Group II; all developed DTH to DNCB or KLH when sensitized 3 days after CY.
Of the 22 patients on the study, 17 have died with a median survival time from CY administration of 5 months (identical for Groups I and II). Five are alive at 6, 6, 10, 19, and 26 months, respectively, after CY treatment.

**DISCUSSION**

The effect of CY on immunity was considered only to be suppressive until the publication of the findings of Maguire and Ettore (14). These authors observed that guinea pigs given CY prior to DNBCB sensitization and contact tested 7 days after sensitization had a much more intense inflammatory response to the test material than did control guinea pigs. The CY-pretreated animals were also different in that their challenge reactions persisted for many days rather than, as in the control animals, fading rapidly after 24 hr. This represented augmentation of the acquisition of DTH rather than its expression since administration of CY before challenge either did not affect DTH or only slightly diminished it. These findings have since been repeatedly confirmed and expanded by a number of investigators using a variety of antigens (1, 7, 12, 17).

In this paper, we demonstrate for the first time that administration of CY can augment the human immune response. Although previous investigations have suggested that cytotoxic drugs can augment human immune function (3), these studies have been limited to measurement of *in vitro* parameters in patients treated with a wide variety of single drugs and drug combinations. We found that pretreatment of advanced cancer patients with CY 3 days before sensitization with KLH markedly augmented the acquisition of DTH as compared with a similar group of patients tested before CY. Although conceivable, it seems very unlikely that the augmented DTH response to KLH was caused by exposure to antigenically unrelated DNBCB. The data also suggest that CY pretreatment enhanced the development of DTH to DNBCB, although with this small sample size the difference did not quite reach statistical significance. It is possible that DNBCB is a weaker immunogen than KLH or that topical application is a less effective way of immunizing than s.c. injection. CY pretreatment did not detectably stimulate or suppress the agglutinating antibody response to KLH.

These results are striking when one considers the clinical and immunological status of the study subjects. All of the patients had advanced metastatic cancer previously treated with 2 or more cytotoxic drugs. Most of them died within 6 months of completing the study. Several of the patients were terminally ill at the time that positive DTH reactions were measured. The mean number of circulating lymphocytes was less than half the normal level. Finally, their pre-CY antibody and DTH responses were markedly depressed, as would be expected in patients with advanced cancer (11). In comparison, we have shown that patients with high risk but surgically excised local or regional melanoma had normal antibody responses to KLH and normal DTH responses to KLH and DNBCB (2).

We chose to test a conventional dose of CY (1000 mg/sq m) because we believed that we were morally obliged to begin with a dose that was known to have antitumor activity. However, this dose is much higher than the immunopotentiating dose found to be effective by most investigators in murine systems (1, 10, 18). It is possible that at this high dose some of the immunopotentiating effect of CY is masked by an immunosuppressive effect and that a lower dose would be more effective. This possibility requires further study.

Experimental evidence suggests that CY augments immunity by depleting or functionally impairing suppressor T-cells. Several investigators (9, 18) have shown that adoptive transfer of normal T-cells abrogates immunopotentiation by CY. Diamantstein *et al.* (8) demonstrated that spleen cells from mice treated with CY showed an increased mitogenic response to dextran sulfate that could be reversed by the addition of normal thymocytes but not by CY-treated thymocytes. Ozer *et al.* (16) have studied the effect of 4-hydroperoxy cyclophosphamide on human blood lymphocytes in *vitro*. They showed that precursors of concanavalin A-inducible suppressor cells are sensitive to this compound at a concentration 5 logs lower than that required to inhibit helper T-cells. If CY has the same selective toxicity for human suppressor T-lymphocytes in *vivo*, then it should be possible to detect a decrease in the number and/or function of these cells in the blood of patients at some time points after its administration.

CY might also modulate the function of other types of suppressor cells. Braun and Harris (3) showed that blood lymphocytes from cancer patients often have increased responsiveness to phytohemagglutinin at certain time points following therapy with a variety of cytotoxic drugs including CY. They observed that this was temporally associated with diminished activity of suppressor monocytes but was not clearly associated with reduction in concanavalin A-inducible suppressor T-cells.

Although the demonstration that CY can augment human immunity is of great theoretical interest, it could also have practical importance. There is evidence that CY augments tumor-associated immunity in experimental animals (9, 10). Administration of CY to tumor-bearing patients followed at the proper interval by administration of tumor antigen could result in the development of a tumor-directed immune response. If this response were strong enough, it might even lead to the destruction of tumor cells and a clinically useful therapeutic effect.

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**REFERENCES**


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