Impairment of Concanavalin A-inducible Suppressor Activity following Administration of Cyclophosphamide to Patients with Advanced Cancer

David Berd, Jr., Henry C. Maguire, Jr., and Michael J. Mastrangelo

Fox Chase Cancer Center, Philadelphia 19111 [D. B., H. C. M., M. J. M.]; University of Pennsylvania School of Medicine, Philadelphia 19104 [D. B.]; Hahnemann Medical College, Philadelphia, 19102 [H. C. M.]; and Temple University School of Medicine, Philadelphia 19140 [M. J. M.], Pennsylvania

ABSTRACT

We have shown that cyclophosphamide (CY) can augment the development of delayed-type hypersensitivity to a primary antigen in patients with advanced cancer. In the present study, we administered CY (1000 mg/sq m) to 19 patients with advanced, metastatic cancer and monitored the compositional and functional changes in their peripheral blood mononuclear cells. Within 2 days of administration of CY, the lymphocyte count fell significantly (mean decrease = 26.0%) and remained significantly depressed through Day 14 with recovery beginning by Day 21. T- and B-lymphocytes were depleted to about the same degree at each time point. Moreover, there was no selective depletion of the Leu 2(+) (suppressor-cytotoxic) or Leu 3(+) (helper-inducer) subsets of T-lymphocytes. Proliferative responses to mitogens (phytohemagglutinin, concanavalin A, pokeweed mitogen) and to allogeneic cells fell significantly within 1 day of administration of CY and continued to be diminished on Day 2. However, these responses recovered to pretreatment levels by Day 3, and, in some cases, exceeded pretreatment levels on Day 7. Concanavalin A-inducible suppressor activity was also diminished on Day 1 (mean decrease, 23.4%) and Day 2 (mean decrease, 39.2%). However, in contrast to the proliferative responses, suppressor activity continued to be significantly impaired on Day 3 (mean decrease, 31.6%) and only partially recovered by Day 7 (mean decrease, 22.1%). Both concanavalin A-inducible suppression and proliferative responses declined again on Days 14 and 21. Thus, between 3 and 7 days after administration of CY, there appeared to be impairment of nonspecific T-cell-mediated suppressor activity of peripheral blood lymphocytes that was not merely a reflection of impaired lymphocyte function in general. This could account for the augmented delayed-type hypersensitivity responses of CY-treated patients.

INTRODUCTION

The alkylating agent CY is a potent immunosuppressive drug (11, 14). However, it has been shown in experimental animals that, under the proper circumstances, this drug can also augment immune responses, especially the development of primary DTH (10, 19).

Recently, we reported that CY can augment human immune responses (3). Thus, patients with advanced cancer developed a significantly greater DTH response to KLH if treated with CY (1000 mg/sq m) 3 days before sensitization with antigen than if they were sensitized without CY pretreatment.

Data from experimental animals suggest that CY immunopotentiates because it is much more toxic to suppressor T-cells than to T-cells that provide help for antibody responses and mediate DTH (9). That hypothesis has just begun to be tested in human systems. Stevenson and Fauci (17) and Ozer et al. (12) have shown that, when normal human peripheral blood T-lymphocytes were exposed to activated forms of CY in vitro and then added to untreated B-cells, PWM-driven proliferation and immunoglobulin production were increased. Furthermore, lymphocytes exposed to activated CY could not be induced by Con A to become nonspecific suppressor cells.

The present study was performed to determine whether administration of CY to patients with advanced cancer had the same effect on the suppressor system as did exposure to activated CY in vitro. We administered CY in a dose that we have shown to cause immunopotentiation of DTH and monitored the compositional and functional changes in peripheral blood lymphocytes.

MATERIALS AND METHODS

Patients. We studied 19 patients with advanced cancer of the following types: malignant melanoma, 9; colon carcinoma, 3; breast carcinoma, 2; ovarian carcinoma, 2; and one patient each with prostate carcinoma, pancreatic carcinoma, and soft tissue sarcoma. The median age was 59 with a range from 32 to 75 years. Ten of the subjects were men. Eighteen of the 19 patients had visceral metastases; one had only skin metastases. Three patients had received limited-field radiation therapy 4, 10, and 15 months prior to entry on the study. All but one patient had received chemotherapy previously, and the median number of prior chemotherapies was 3 (range, 0 to 6). No patients received chemotherapy within 4 weeks of entry on this study. No patients were receiving corticosteroids or other drugs with documented immunomodulating effects.

CY. This was given as a 15-min i.v. infusion. The dose was 1000 mg/sq m.

Peripheral Blood MNC. Blood was collected in heparinized tubes before and at various times after administration of CY. Leukocytes were counted with a Coulter Counter. Differential counts were made on Giemsa-stained smears by counting at least 200 cells on 2 different slides. MNC were separated by density gradient centrifugation on Ficoll-metrizoate (4); suspended in freezing medium, consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), penicillin, streptomycin, 10% pooled human AB-positive serum (Biocell Laboratories, Carson, CA), and 10% dimethyl sulfoxide; frozen in a controlled-rate freezer; and stored in liquid nitrogen. When needed for testing, MNC were thawed rapidly, diluted slowly to gradually reduce the concentration

1. Supported by USPHS Grant CA32123 and by an appropriation from the Commonwealth of Pennsylvania.

2. To whom requests for reprints should be addressed.

3. The abbreviations used are: CY, cyclophosphamide; Con A, concanavalin A; DTH, delayed-type hypersensitivity; FITC, fluorescein isothiocyanate; IodUr, iodo-deoxyuridine; KLH, keyhole limpet hemocyanin; MLR, mixed-lymphocyte response; MNC, mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RITC, rhodamine isothiocyanate.

Received July 21, 1983; accepted November 30, 1983.

MARCH 1984

1275
of dimethyl sulfoxide, treated with Tris-buffered ammonium chloride to lyse erythrocytes, and washed twice.

**Determination of MNC Composition.** The following antibody reagents were used: FITC-conjugated anti-Leu-4 (Becton-Dickinson, Mountainview, CA), which binds to all peripheral T-cells; FITC:anti-Leu-3, which binds to a subset of T-cells that provide help for antibody production; FITC:anti-Leu-2, which binds to a subset of T-cells that have suppressor and cytotoxic functions (6); FITC:anti-human immunoglobulin (Cappel Laboratories, Lancaster, PA); anti-Mo 2 (Bethesda Research Laboratories, Bethesda, MD), which binds to monocytes (20); and RITC-conjugated Fab fragment goat anti-mouse immunoglobulin as a second-step reagent for the anti-Mo antibody (Cappel Laboratories). All reagents were freed of aggregates by centrifugation in an Air Fuge (Beckman, Irvine, CA) each time they were used.

All MNC collected serially from an individual patient were thawed and analyzed on the same day when possible. If the number of samples was too large to permit this, then the analysis of the samples of a patient was spread over 2 days. Each MNC sample was divided into 6 aliquots. One aliquot was used for sizing by light scatter analysis and determination of viability by ethidium bromide exclusion; the proportion of dead cells, as indicated by red fluorescence, was always less than 5%. The other aliquots were stained in a 3-step procedure. (a) All samples were incubated on ice with anti-Mo 2. (b) They were then washed and incubated on ice with RITC anti-mouse immunoglobulin. (c) They were washed and incubated with one of the following: diluent; FITC:anti-Leu 4; FITC:anti-Leu 3; FITC:anti-Leu 2; or FITC:anti-human immunoglobulin. In preliminary experiments, mouse IgG was added before incubation with the FITC-conjugated reagents to block residual binding sites, but this step was found to be unnecessary.

The proportion of cells binding each of the fluorochrome-conjugated antibodies was determined by a fluorescence-activated cell sorter (FACS II; Becton-Dickinson). Gates were set so that cells exhibiting red fluorescence, i.e., binding anti-Mo 2 and the RITC second-step reagent, were excluded from analysis. In this way, monocytes that bound the antibodies nonspecifically or by virtue of passively absorbed surface immunoglobulin were not counted as T- or B-lymphocytes. The number of T-cells (Leu 4+), helper-inducer T-cells (Leu 3+), suppressor-cytotoxic T-cells (Leu 2+), and B-cells (immunoglobulin positive) was determined by multiplying the percentage of stained cells by the total number of lymphocytes.

**Lymphoproliferative Responses.** All of the samples collected serially from an individual patient were tested on the same day. MNC prepared as described above were suspended in culture medium, consisting of RPMI 1640, penicillin, streptomycin, 10% pooled human AB-positive serum, 10^{-3}M 2-mercaptoethanol, and 0.1 μg 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 1 x 10^5 cells were added to microtiter wells (Costar, Cambridge, MA). Then, one of the following stimulants was added: PHA (Wellcome Reagents, Beckenham, England; optimal concentration, 1.0 μg/ml; suboptimal concentration, 0.1 μg/ml); Con A (Pharmacia Fine Chemicals, Piscataway, NJ; optimal concentration, 10 μg/ml); PWM (Grand Island Biological Co.; optimal dilution, 1:50); or 10^6 mitomycin C-treated allogeneic MNC (MLR). The latter consisted of a mixture from 3 normal donors, and the same mixture was used throughout these experiments. Some microtiter wells were supplemented with indomethacin (1 μg/ml; Sigma Chemical Co., St. Louis, MO). The cultures were incubated in a 37° incubator in a 5% CO2 atmosphere for 3 days (mitogens) or 6 days (MLR). Then, they were pulsed with ^3H]-labeled IdUrd (New England Nuclear, Boston, MA) for 6 hr and harvested with a MASH II harvesting device (MA Bioproducts, Walkersville, MD). [^3H]-IdUrd uptake was measured in a γ counter, and the mean of triplicate wells was calculated; the triplicates never varied by more than 20%.

**Con A-inducible Suppressor Activity.** This was performed by a modification of the method of Shou et al. (15). MNC (2 x 10^5) were incubated in 1 ml of culture medium with or without Con A (25 μg/ml) in plastic tubes in a 37°, 5% CO2 incubator for 3 days. Then, they were washed and exposed to mitomycin C (Sigma; 25 μg/ml) for 30 min in a 37° water bath. After 3 washes, they were suspended in culture medium, and either 1 x 10^6 or 5 x 10^6 cells were added to 1 x 10^6 indicator cells" in microtiter wells. The latter were MNC from a normal donor, and the same donor was used in all the experiments described herein. Then, PHA was added to all the wells at a predetermined suboptimal concentration (0.1 μg/ml). The mixtures were incubated at 37° in 5% CO2 for 3 days and then pulsed and harvested as described above. The percentage of suppression was calculated as follows.

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\frac{100 \times (cpm (/ + UN) - cpm (/ + Con A))}{cpm (/ + UN)}
\]

where cpm (/ + UN) are the uptake of [^3H]-IdUrd by indicator cells in the presence of unstimulated patient's cells, and cpm (/ + CON A)are the uptake by indicator cells in the presence of Con A-stimulated patient's cells. Allogeneic indicator cells were used because they allowed for better standardization of the assay. Moreover, it has been shown repeatedly that Con A-inducible suppression is not HLA restricted (12, 15). Finally, Ozer et al. (12) used an allogeneic mixture in their studies of the effect of CY in vitro on Con A-inducible suppression.

**Analysis of Data.** For each patient and for each parameter, we calculated the percentage of change between the pre-CY sample and each post-CY sample. Then, for a given parameter, we determined whether the mean percentage of change of the whole group was significantly different from zero at each time point by using the Student t test for nonindependent variables (2 tailed). This approach avoids the potential problem of mistaking spontaneous fluctuations for CY-induced changes.

**RESULTS**

**Effect of CY on Circulating Leukocytes.** As expected, administration of CY (1000 mg/sq m) resulted in a fall in circulating neutrophils. Before CY, the mean neutrophil count per cu mm was 7347 ± 639 (S.E.). There was no significant change at 1, 2, or 3 days after CY, but at 7 and 14 days, the neutrophil counts were decreased by means of 28.9 and 50.0%, respectively (p < 0.01). In contrast was the kinetics of the fall in circulating lymphocytes after CY (Chart 1). Before CY, the mean lymphocyte count per cu mm was 1424 ± 156. Lymphocyte numbers decreased by a mean of 26.0% within 2 days after CY and remained significantly depressed through Day 7, with recovery beginning on Day 14.

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**Chart 1.** Change in the number of circulating lymphocytes (C), T-cells (T), and B-cells (B) after administration of CY. Columns, mean percentage of change from pre-CY values. *, p < 0.05; **, p < 0.01; others, not significant.
Effect of CY on Lymphocyte Composition. Before CY, the mean numbers of lymphocytes in the various subpopulations were as follows (per cu mm): B-lymphocytes (immunoglobulin positive), 139 ± 29; T-lymphocytes (Leu 4+), 1140 ± 130; T-helper-inducer subset (Leu 3+), 775 ± 95; T-suppressor-cytotoxic subset (Leu 2+), 394 ± 54.

Following CY, the number of T- and B-lymphocytes decreased by Day 2, remained depressed through Day 14, and did not begin to recover until Day 21 (Chart 1). T- and B-lymphocytes were depleted to about the same degree at each time point. Unexpectedly, we observed an increase (mean, 22.3%) in the number of B-cells 1 day after CY; this did not quite reach statistical significance (0.05 < p < 0.10). Whether or not this is a biologically significant finding is uncertain.

The effect of CY on the 2 T-cell subsets is shown in Chart 2. There was no selective depletion of suppressor-cytotoxic (Leu 2+) or helper-inducer (Leu 3+) T-cells.

Lymphoproliferative Responses. Before CY, the proliferative responses to optimal concentrations of mitogens were as follows (cpm/10^5 cells plated): PHA, 118,586 ± 11,303; PWM, 35,131 ± 4,953; and Con A, 47,081 ± 6,439.

The mean changes in proliferative response to optimal concentrations of mitogens following CY are depicted in Chart 3. A scattergram showing the changes in PHA response of individual patients is presented in Chart 4. The responses to the 3 mitogens were similarly affected at all time points. Mitogen responses fell significantly within 1 day of administration of CY and continued to be diminished on Day 2. However, these responses recovered to pretreatment levels by Day 3 and, for PWM and Con A, were slightly, but not significantly, above pretreatment levels by Day 21.

7. There appeared to be a secondary fall in mitogen responses on Days 14 and 21.

We considered the possibility that the lymphoproliferative response of a suboptimal concentration of mitogen might be more sensitive to CY-induced changes. Therefore, we also tested the response of MNC to a suboptimal concentration of PHA, 0.1 μg/ml (one-tenth of the optimal dose). Before CY, the mean response was 51,853 ± 5,859. One day after CY, the mean response decreased by 12.6% (p < 0.01) but recovered to pretreatment levels by Day 2 and was unchanged on Days 3 and 7. In 4 patients, the response to suboptimal PHA rose 25 to 50% above pretreatment levels on Day 3. However, we could not be certain whether, in these individuals, this truly represented "rebound overshoot" (5) or merely spontaneous fluctuation. As with the optimal concentrations of mitogen, there was a secondary fall in...
responsiveness on Days 14 and 21 (mean decrease, 11.4 and 9.7%, respectively), but these decreases were not statistically significant.

MLRs followed the same pattern as did the proliferative responses to mitogen. The MLR was decreased by a mean of 25.4% (p < 0.01) on Day 2, returned to pretreatment levels on Days 3 and 7, and fell again on Day 14 by a mean of 25.3% (p < 0.01).

**Con A-inducible Suppressor Cells.** The generation of non-specific suppressor cells by incubation with Con A was very sensitive to CY treatment. We added the patients' mitomycin-treated MNC (the putative suppressor cells) to normal, allogeneic MNC (the indicator cells) at a suppressor:indicator ratio of 1:1 or 0.5:1. Prior to CY administration, the percentage of Con A-inducible suppression, calculated as described in "Materials and Methods," was 64.5 ± 1.9 (S.E.) at the 1:1 ratio and 53.2 ± 3.7 at the 0.5:1 ratio. Chart 5 shows the mean percentage of Con A-inducible suppression for the group of 19 patients at a 0.5:1 suppressor:indicator ratio before CY (Day 0) and at 1, 2, 3, 7, 14, and 21 days after CY. The same data are depicted in another manner in a scattergram (Chart 6), which shows the percentage of change in the amount of Con A-inducible suppression from the pre-CY value at each time point for individual patients.

As shown in Charts 5 and 6, Con A-inducible suppression declined by Day 1 after CY and continued to be significantly diminished on Day 2. However, in contrast to the proliferative responses which had recovered to pretreatment levels, Con A-inducible suppressor activity continued to be significantly impaired on Day 3 and only partially recovered on Day 7. As with the proliferative responses, there was a secondary fall on Days 14 and 21. Although changes were most strikingly demonstrated when the suppressor:indicator cell ratio was 0.5:1, they were also observed when the ratio was 1:1 (mean change at Day 3 after CY, -13.2%; p < 0.05).

**Monocytes.** The changes in the number of circulating monocytes varied greatly from patient to patient; at no time point was the mean of the changes statistically significant. This large variation was noted whether monocytes were enumerated by morphological criteria on Giemsa smears or by analysis of MNC with the anti-Mo 2 monoclonal antibody. The mean decrease in circulating monocytes was less than 10% at all time points, except Day 7, at which time the monocyte count had decreased by a mean of 28.3% ± 16.0 (p < 0.10).

We also sought to determine whether CY resulted in diminished function of indomethacin-sensitive suppressor cells, which are generally considered to be monocytes (5). We reasoned that if CY administration resulted in depletion or impairment of suppressor monocytes, then the ability of indomethacin to augment the lymphoproliferative response might decrease following CY. However, in only 2 of 9 pre-CY MNC samples did the addition of indomethacin augment the proliferative response to a suboptimal concentration of PHA, and in both cases, the augmentation was modest (19.9 and 20.2%). Moreover, following CY, there was no change in indomethacin-sensitive suppressor activity in any of the MNC samples.

**Response to CY and Survival.** Only one patient exhibited tumor regression following CY; it was partial and of brief duration. All 19 patients have died with a median survival time of 2 months (range, <1 to 12 months). Patients who lived more than 2 months tended to exhibit a greater fall in Con A-inducible suppression after CY than did patients who lived 2 months or less, but the difference was not statistically significant (proportion of patients with a 50% or greater decrease in Con A-inducible suppression on Day 3 or 7: survivors > 2 months = 5 of 8; survivors 2 months or less = 1 of 8; 0.10 > p > 0.05; 1 tailed; Fisher's exact test). For all other immunological parameters, the magnitude of CY-induced alterations showed no tendency to correlate with survival time.
DISCUSSION

CY is generally regarded as a potent immunosuppressive drug in both experimental animals (11) and in humans (14). Yet, the original observation of Maguire and Ettore (10) and the many confirmatory reports that followed (18) have led to the realization that CY can augment immune responses in experimental animals as well as suppress them. The critical factor determining whether CY depresses or potentiates an immune response is the timing of administration of CY and antigen. For example, in one system (19), the administration of CY 2 days before sensitization with antigen increased the contact hypersensitivity response 3- to 4-fold, whereas administration of CY 2 days after sensitization completely suppressed it.

We have extended these findings recently to humans (3) with advanced cancer. Pretreatment of such patients with CY (1000 mg/sq m) 3 days before injection of a sensitizing dose of KHL augmented significantly the development of DTH to that antigen. The median DTH response of patients given KHL without pretreatment was 0 (4 of 11 positive), as compared to 19 mm (11 of 11 positive) in CY-pretreated patients.

The leading hypothesis to explain immunoaugmentation by CY is that CY selectively depletes or functionally impairs suppressor T-cells. This idea is supported by data from experimental animals (8, 9, 13) and, to a lesser extent, by data from human in vitro systems (12). The present study was undertaken to determine whether the administration of CY in a manner known to be immunopotentiating results in compositional or functional changes in peripheral blood lymphocytes that reflect an impairment of the suppressor system.

The most important observation of this study was the impairment of Con A-inducible suppressor activity following the administration of CY. Such activity fell within 24 hr of administration of the drug and remained significantly depressed on Days 2 and 3. The reduction of suppressor activity on Day 7 was of borderline significance, which suggests that recovery was beginning to occur at that time point. Although lymphoproliferative responses to mitogens and alloantigens also declined for 24 to 48 hr after CY, those responses returned to pretreatment levels by Day 3 and remained unchanged through Day 7. Both Con A-inducible suppressor activity and proliferative responses declined again on Days 14 and 21. Thus, between Days 3 and 7, there appeared to be impairment of nonspecific T-cell-mediated suppressor activity of peripheral blood lymphocytes that was not merely a reflection of impaired lymphocyte function in general.

This impairment of suppressor function was not associated with selective depletion of T-cells bearing what is thought to be the suppressor phenotype. The administration of CY resulted in a rapid and prolonged depletion of circulating lymphocytes. As reported by others (1, 16), T- and B-lymphocytes were about equally affected. However, Leu 2(+) T-cells, a subset that has been reported to mediate suppressor activity (6), were depleted to about the same extent as were Leu 3(+) T-cells, which are considered to be helper-inducer T-cells (6). The simplest explanation for this finding is that the precursors of Con A-inducible suppressor cells represent only a small percentage of the Leu 2(+) population. Therefore, selective depletion of those precursors would not result in a detectable depletion of that population. Alternatively, this finding could be explained by some recent studies suggesting that cells bearing what was originally described as the helper phenotype [Leu 3(+) or OKT4(+)] may function as suppressor cells or as inducers of suppressor cells (12, 18). It is possible that CY selectively depletes such a Leu 3(+) subset; this subset might be identified with one of the newly developed monoclonal reagents (7).

In humans, following the administration of CY, cytotoxic compounds appear in the circulation by 30 min, reach peak levels at 2 to 3 hr, and are detectable for about 24 hr (2). Exposure to high concentrations of these compounds during this initial period could result in the killing of many circulating lymphocytes and sublethal damage to many others; this would be manifest by a fall in the lymphocyte count and a generalized impairment of lymphocyte function during the first 24 to 48 hr. The recovery of the lymphoproliferative responses that we observed on Day 3 was probably due to repair of sublethal damage, although replenishment of the circulating lymphocyte pool by lymphocytes from other body pools may have contributed. The sustained impairment of Con A-inducible suppressor activity could have been due to selective killing of a "presuppressor" cell (12) or to a decreased ability of such cells to repair sublethal CY-induced damage. Finally, the reduction in the number of circulating lymphocytes and the generalized impairment of lymphocyte function that we observed on Days 14 and 21 could have been due to toxic effects on the precursor cells that are needed to maintain the functional circulating lymphocyte pool. Our data validate in an in vivo setting the in vitro studies reported by Ozer et al. (12). These investigators observed that a 1-hr exposure of normal human T-cells to very low concentrations of 4-hydroxyxycyclophosphamide (as low as 10^-9 nmol) prevented the development of Con A-inducible suppressor cells. However, treatment of T-cells after they had been induced by Con A had no effect on their suppressor function. Ozer et al. (12) as well as Stevenson and Fauci (17) have shown that the suppressor cells that modulate PWM-driven immunoglobulin are also selectively sensitive to CY.

This observation of impairment of suppressor T-cell function 3 days after administration of CY fits well with our original observation that the administration of a primary antigen to cancer patients 3 days after CY results in augmentation of DTH. CY could result in augmentation of DTH by depleting the precursors of antigen-specific suppressor cells, just as it depletes the precursors of Con A-inducible suppressor cells. However, other explanations for CY-induced immunopotentiation must also be considered. Braun et al. (5) have suggested that cytotoxic drugs can selectively inhibit suppressor monocytes. We were unable to demonstrate significant depletion of circulating monocytes at any time point after administration of CY, but we could not test for changes in indomethacin-sensitive, monocyte-mediated suppressor function because so few of our patients demonstrated such suppression before receiving CY. This may have been due to the use of cryopreserved, rather than freshly separated, monocytes. Finally, there could be CY-induced alterations in the composition and function of lymph node lymphocytes that are critical to the augmentation of DTH to a cutaneously injected antigen and that are not detected in peripheral blood cells. Investigation of such hypotheses should lead to a better understanding of not only the mechanism of action of CY but also human immunoregulatory function in general.

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*Cancer Res* 1984;44:1275-1280.

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