Recovery of the in Vivo Cytotoxic T-Cell Response in Cyclophosphamide-treated Mice by Injection of Mixed-Lymphocyte-Culture Supernatants1

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ABSTRACT

Mice given injections of high antileukemic doses of cyclophosphamide lost the capacity to generate cytotoxic T-cells in vivo to allogeneic tumor cells. These low responses were not due to the elimination of cytotoxic T-lymphocyte precursors because normal cytotoxic responses were obtained in vivo after cyclophosphamide treatment by injection of helper factor derived from mixed-lymphocyte-culture supernatants.

INTRODUCTION

CY2 has been used effectively in the control of neoplasia in humans and experimental animals (2, 6, 10). In mice, high single doses of this drug have been shown to be more effective against L1210 leukemia than were the same or even larger doses given repeatedly (10, 11, 20). Single high doses of CY, however, inhibit cytotoxic T-cell development in vitro and in vivo (1, 7, 8, 17, 19).

We have shown previously that the generation of CTL directed toward allogeneic tumor cells is inhibited by CY in a dose-dependent manner. This reduction of CTL activity is not due to the depletion of cytotoxic T-lymphocyte precursors but to helper cells required for CTL development (14). This CY-sensitive helper cell activity can be replaced by the addition of Lyt 1+2~ helper cells to an in vitro CTL culture system (15) or by helper factors derived from MLC supernatants (16).

In this report, these studies have been extended to show that mice immunized with a CY-resistant tumor line (L1210-CYR) do not generate CTL in vivo after high-dose CY therapy. This low in vivo CTL response, after CY injection, can be restored to normal levels by injection of MLC supernatants containing T-cell-derived helper factor.

MATERIALS AND METHODS

Experimental Animals. Male C57BL/6 (H-2b) and male DBA/2 (H-2d) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Cell Lines. P815-X2 (H-2b) mastocytoma cells and EL4 (H-2b) lymphoma cells were maintained by weekly i.p. passage in syngeneic DBA/2 and C57BL/6 mice, respectively. A L1210 cell line (H-2b) and a CY-resistant L1210 subline (L1210-CYR) were maintained by weekly i.p. passage in DBA/2 mice. Mice inoculated with the L1210-CYR cell line showed no increase in mean survival time after CY therapy, whereas DBA/2 mice inoculated with L1210 cells showed a 100% increase in mean survival time after CY therapy. C11B (H-2b) myeloma cells of C3H origin were maintained by in vitro passage in RPMI supplemented with 10% heat-inactivated FCS.

Preparation of MLC Supernatants. Spleen cells from C57BL/6 mice (2.5 × 107) were mixed with irradiated (1000 R) spleen cells from DBA/2 mice (2.5 × 107) in 20 ml of RPMI supplemented with 10% heat-inactivated (56°, 45 min) FCS and 5 × 10–4 M 2-mercaptoethanol. The cultures were incubated for 14 days in Corning No. 251002 flask at 37° in a humidified atmosphere with 5% CO2. The responder cells were then harvested, washed, counted, adjusted to 108/ml, and restimulated with irradiated (1000 R) DBA/2 spleen cells (4 × 106/ml) in 10-ml aliquots in Falcon No. 3012 flasks for 24 hr. The last 24 hr culture was set up in RPMI without FCS. After 24 hr, the cultures were centrifuged, and the supernatant was concentrated 8- to 10-fold with Amicon YM-10 filters prior to injection. The concentrated supernatants were frozen in aliquots at −18°.

CY. The CY-sodium chloride preparation (Mead Johnson, Evansville, Ind.) was dissolved in distilled water to a concentration of 20 mg/ml. Individual mice were weighed and CY was administered by i.p. injection. There was consistently a 50 to 75% reduction in lymph node cellularity after CY treatment.

In Vivo CTL Generation and Assay. C57BL/6 mice (3 to 12/group) were given injections of allogeneic tumor cells i.m. in the hind flank. Optimal concentrations for CTL development were 5 × 106/flank for L1210 and L1210-CYR leukemia cells. CY was administered at 180 mg/kg 24 hr after tumor cell inoculation. MLC supernatants were injected twice a day i.m. (0.1 ml) at the site of the inguinal node. Seven days after the tumor implant, the inguinal node was removed and cells were teased into suspension. Cells were washed, counted, and mixed with 51Cr-labeled target cells in microculture plates and incubated at 37° in a humidified atmosphere with 5% CO2. After 5 hr, the effector cells were centrifuged and samples (0.1 ml) of each culture well were analyzed in a Nuclear Chicago γ counter. The percentage of specific 51Cr release was calculated in the following manner:

\[
\text{% of } ^{51}\text{Cr release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100
\]

Total releasable counts were determined by dilution of 51Cr-labeled target cells in distilled water and were always >85% of the total input.

RESULTS AND DISCUSSION

Initial experiments were designed to compare the immunizing capacities of the L1210, L1210-CYR, and P815 tumor cell lines in C57BL/6 mice and their susceptibility to suppression by different doses of CY 1 and 3 days after tumor implant. All 3 tumors were comparable in their ability to immunize C57BL/6 mice, and all 3 systems were equally susceptible to immu-
The experiments outlined in this report are unique because we can specifically study immunosuppression induced by CY, therefore affecting its immunization capacity. In this respect, we chose to study only the L1210-CYR cell line because of its resistance to cyto reduction by CY. This eliminated the possibility of drug-induced reduction of the antigenic load by CY, therefore affecting its immunization capacity. In this respect, we can specifically study immunosuppression induced by CY without affecting the immunogen.

In a previous report using an in vitro system (16), we have shown that helper factor derived from MLC supernatants restores CTL activity to CY-treated spleen cells. Recently, Wagner et al. (23) and Stötter et al. (21) have shown that helper factor (interleukin 2) is able to circumvent the requirement for thymus-derived T-cells in nu/nu mice. These mice can be induced to produce antibody to heterologous erythrocytes and alloreactive CTL after antigen and helper factor administration. The experiments outlined in this report are unique because we have shown that the CTL response can be rescued after high-dose CY therapy by the in vivo administration of helper factor. Table 1 shows the results of 2 experiments in which helper factor was injected in vivo after CY administration. Mice bearing the L1210-CYR tumor alone demonstrated a normal CTL response, while those mice given injections 24 hr after tumor implant with CY had a poor CTL response. Mice given injections of CY and MLC supernatant showed a recovery in CTL development (Table 1, Experiment 1). We have determined that the MLC supernatant must be injected for at least the first 3 days after tumor implant for full recovery (Table 1, Experiment 2).

It has been shown that MLC-activated lymphocytes produce interferon (13) and that interferon stimulates natural killer cell activity (9). In order to test whether the restorative cytotoxic activity after the administration of MLC supernatants resulted from null cells or Thy 1.2 (β-bearing)-derived T-lymphocytes, effector cells from the draining lymph nodes were treated with anti-Thy 1.2 and complement before analysis in the 

It was possible, however, that the cytotoxic precursor cells stimulated by MLC supernatants were nonspecific effector cells and would not show target specificity. The following experiment was designed to test this possibility. Three groups of C57BL/6 mice were inoculated with L1210-CYR cells. One group was left untreated, a second group received CY (180 mg/kg) 24 hr later, and a third group received CY and MLC supernatant. On the seventh day, inguinal nodes were removed from each group, teased into suspension, and mixed with 3 groups of 

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C57BL/6 mice were inoculated with L1210-CYR (5 x 10⁶/mouse) and were left untreated (B6) or given injections of CY (B6-CY) 24 hr later (180 mg/kg).

Lymph node effector cells were treated with either complement alone or anti-Thy 1.2 and complement. Five hr ⁵¹Cr release assay: lymphocyte/target cell ratio of 50/1; spontaneous release, 13.1%.

C⁵⁺, complement; #, anti-Thy 1.2.

The therapeutic effectiveness of CY as an anticancer agent is compromised by its strong immunosuppressive properties (10). Several studies have suggested that the use of CY as an antitumor drug depends upon intact host immunity (3, 4, 12, 18).

In this report, we have shown that in a strictly in vivo system a powerful antineoplastic drug, CY, spares most cytotoxic T-lymphocyte precursors from cyto reduction. This has been confirmed in our earlier studies using in vitro assays (14-16) and by Taswell et al. (22) using in vitro-limiting dilution analysis. This study extends our previous results by showing that a second signal for the generation of CTL is lacking in CY-treated mice and can be replaced by in vivo therapy with MLC-derived helper factor. Although the specific chemical nature of the

Table 2

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B6 x DBA (1000 R) MLC supernatant injected i.m. twice a day (0.1 ml) for 3 days.

Lymph node effector cells were treated with either complement alone or anti-Thy 1.2 and complement.

Five hr ⁵¹Cr release assay: lymphocyte/target cell ratio of 50/1; spontaneous release, 13.1%.

C⁺, complement; #, anti-Thy 1.2.

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B6 x DBA (1000 R) MLC supernatant injected i.m. twice a day (0.1 ml) for 3 days.

Five hr ⁵¹Cr release assay: lymphocyte/target cell ratio of 50/1.

Spontaneous release, 13%; percentage of total releasable counts, 45.1.

Spontaneous release, 9%; percentage of total releasable counts, 84.9.
helper factor in MLC supernatants that restores CTL activity in vivo is unknown, studies have shown that interleukin 2 may be the responsible entity (21,23). A recent report by Fidler (5) has shown that a lymphokine, macrophage-activating factor, can be delivered functionally and specifically in vivo by liposomes. We believe that certain biological mediators, such as T-cell-derived helper factors, that are eliminated by immunosuppressive anticancer agents may also be supplied exogenously by similar means in clinical situations so that the defenses of the host may be more properly aligned to eliminate residual antigenic tumor cells and, of equal importance, pathological infections.

REFERENCES


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