Some Advantages of Curing Mice Bearing a Large Subcutaneous MOPC-315 Tumor with a Low Dose Rather Than a High Dose of Cyclophosphamide

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ABSTRACT

Mice bearing a large s.c. MOPC-315 tumor can be cured by a dose of cyclophosphamide (CY) ranging from 15 to 200 mg/kg. However, the low (15 mg/kg) and the high (200 mg/kg) doses mediate tumor eradication via different mechanisms. Tumor eradication by the low dose of drug requires the cooperation of the toxic effect of the drug and T-cell-dependent antitumor immunity. On the other hand, tumor eradication by the high dose of drug does not require the participation of antitumor immunity but depends primarily on the tumoricidal activity of the drug. Spleen cells from tumor-bearing mice treated with the low dose of CY exhibit an augmented antitumor immune potential, whereas spleen cells from tumor-bearing mice treated with the high dose of CY exhibit suppressed antitumor immune potential. More importantly, tumor-bearing mice treated with the low dose of drug are able to reject a challenge with 300 times the minimal lethal tumor dose given 1, 6, or 31 days after CY therapy, whereas mice treated with the high dose of drug are unable to reject such a challenge given within the same time intervals after CY therapy. Moreover, when mice bearing a large tumor are treated with the high dose of CY and subsequently challenged again with tumor cells to establish a Day 4 nonpalpable tumor, this tumor is less responsive to cure by combined chemoinmunotherapy than is a Day 4 nonpalpable tumor established in normal mice. Thus, although the high dose of CY can cure most mice bearing a large-size MOPC-315 tumor, it not only does not result in antitumor immunity, but it actually reduces the effectiveness of chemoinmunotherapy for a second tumor challenge. In contrast, mice cured with the low dose of CY exhibit long-lasting potent antitumor immunity.

INTRODUCTION

Antitumor immunity can facilitate the effectiveness of cancer chemotherapy (10, 21, 23, 30, 33). This might be mediated via the following mechanisms: (a) the drug might reduce the tumor burden to a level whereby the existent host antitumor immunity can eliminate the remaining tumor cells (10); (b) the drug might slow tumor growth long enough to allow a potent host antitumor immune response to develop (10); (c) the drug might render residual tumor cells more susceptible to immune lysis (6, 35); (d) the drug might render tumor cells more immunogenic, thereby providing a superior stimulation for the development of host antitumor immunity (1, 14, 15); and (e) the drug might act as an immunomodulator leading to the elimination of suppressor cells (20, 31), thereby allowing the generation and/or expression of augmented levels of antitumor immunity (20).

We have shown recently that the dose of drug required for the cure of MOPC-315 tumor-bearing mice can be decreased if antitumor immunity exhibited by the tumor-bearing mice is increased. Accordingly, a low dose of CY, 15 mg/kg, which is not curative for mice at an early stage of MOPC-315 tumor growth, cured most mice bearing a nonpalpable Day 4 tumor when administered in conjunction with adoptively transferred immune spleen cells (29). Moreover, the same low dose of CY (15 mg/kg), which was not curative for mice at an early stage of tumor growth due to the presence of insufficient levels of antitumor immunity (29), was curative for mice bearing a large (20 to 25 mm; Days 10 to 16) tumor due to the participation of potent host antitumor immunity in tumor eradication (21).

Mice bearing a MOPC-315 tumor can be cured with a high dose of CY (200 mg/kg) (20). The curative effectiveness of the high dose of CY for mice bearing a nonpalpable tumor was due primarily to the tumoricidal activity of the metabolites of the drug (29). However, it is not known whether the curative effectiveness of the high dose of CY for mice bearing a large tumor is also due solely to the tumoricidal effect of the metabolites of the drug. Since it is a common practice in clinical oncology to treat patients with relatively high doses of chemotherapeutic drugs, we evaluated, in an animal tumor model, whether high-dose CY therapy offers any curative advantage over low-dose CY therapy. In addition, we analyzed the mechanism of eradication of a large tumor by a low or a high dose of CY.

MATERIALS AND METHODS

Spleen Cell Suspensions. Cell suspensions were prepared from spleens of normal female BALB/c mice (8 to 12 weeks old; Laboratory Supply Co., Indianapolis, Ind.) or from CY-treated BALB/c mice bearing s.c. MOPC-315 tumors. In any individual experiment performed, the spleens used in each group were obtained from at least 3 but usually 5 mice. Single-cell suspensions were prepared by mechanical disruption (27), and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 95%.

Tumors. The weakly immunogenic MOPC-315 plasmacytoma (34) was maintained by serial s.c. inoculation in syngeneic BALB/c mice. Routinely, mice were inoculated with 1 x 10^6 viable MOPC-315 cells, a dose which is 300 times greater than the minimal lethal tumor dose and kills the mice in 21 ± 1 (S.E.) days. Single-cell suspensions were prepared by mechanical disruption (27), and the viability as determined by trypan blue dye exclusion (0.4%) always exceeded 85%.

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3 The abbreviations used are: CY, cyclophosphamide; ATS, rabbit anti-mouse thymocyte serum; TuB-15 mice, mice bearing large tumors and treated with CY (15 mg/kg); TuB-200 mice, mice bearing large tumors and treated with CY (200 mg/kg).

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Chemotherapy. CY (Cytoxan; Mead Johnson and Co., Evansville, Ind.) was dissolved in sterile distilled water (to a concentration of 20 mg/ml) and further diluted in minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.). CY therapy given in these experiments consisted of a single i.p. injection of either 15 or 200 mg/kg in 0.5 ml. Tumors were measured 3 times weekly with vernier calipers, and mice that had no detectable tumors 60 days after chemotherapy were considered to be cured. Each experiment was repeated 2 to 4 times.

In Vitro Immunization. The in vitro generation of antitumor cytotoxicity was performed by a modification of the method of Burton et al. (9), as we have described previously (27, 28). Briefly, responder spleen cells $(75 \times 10^6)$ were cultured with mitomycin C-treated $(50 \mu g/ml$ for 30 min) MOPC-315 stimulator tumor cells $(2.5 \times 10^6)$ in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 5% fetal calf serum, 1% nonessential amino acids, 50 units penicillin per ml, and 50 $\mu g$ streptomycin per ml (Grand Island Biological Co.). The cultures were incubated at $37^\circ$ in 5% CO$_2$ in air for 5 days, the time required for the in vitro generation of optimal levels of antitumor cytotoxicity (27, 28).

In Vitro Antitumor Cytotoxicity Assay. Cell-mediated lysis was determined as we have described previously (27), utilizing the 3.5-hr in vitro $^{31}$Cr release assay (8). The percentage of specific $^{31}$Cr release was assessed by the following formula:

$$\text{% of specific } ^{31}\text{Cr release} = \frac{T - C}{M - C} \times 100$$

where $T$ is the percentage of release with test spleen cells, $C$ is the percentage of spontaneous release by the target cells alone which ranged between 14 and 18%, and $M$ is the percentage of maximal $^{31}$Cr release obtained by 3 cycles of freezing and thawing, which ranged between 78 and 81%. Each experiment was performed 2 to 4 times. The level of antitumor cytotoxicity is presented as the mean $^{31}$Cr release of triplicate samples. Variations in the $^{31}$Cr release between individual samples rarely exceeded 3% of the mean. All points that differed by 8% or more $^{31}$Cr release were found to be significantly different by Student’s $t$ test ($P < 0.05$).

Antithymocyte Serum. ATS was obtained from Microbiological Associates (Walkersville, Md.) and stored at $-20^\circ$ prior to use. Mice were given 3 i.p. injections of 0.25 ml each on Days 2, 4, and 7 post-CY therapy. This protocol of ATS treatment was shown to abolish the ability of spleen cells to proliferate in response to a T-cell mitogen (phytohemagglutinin) and to generate primary or secondary cellular antitumor immunity, whereas it only minimally reduced the ability of the spleen cells to proliferate in response to a B-cell mitogen (lipopolysaccharide) (21, 29).

RESULTS

Comparison of the Kinetics of Regression of a Large MOPC-315 Tumor following Treatment with a Low or a High Dose of CY. BALB/c mice were given a s.c. injection of $1 \times 10^6$ MOPC-315 tumor cells. Twelve days after tumor inoculation, when the tumors had reached 20 mm in diameter, the mice were treated with a single i.p. injection of either 15 or 200 mg CY per kg, and subsequently, the tumor size was monitored periodically (Chart 1). Following CY therapy with 15 mg/kg, tumor size did not change for the first 2 days. However, a significant reduction in tumor size was observed 5 days after chemotherapy, and the tumors continued to decrease in size thereafter, resulting in the complete regression of the tumors by Day 9 after chemotherapy. In contrast, following CY therapy with 200 mg/kg, a significant reduction in tumor size was already seen in the first 2 days after CY therapy, and the tumors continued to decrease in size, resulting in complete tumor regression by Day 7. Thus, treatment of mice bearing a large tumor with 200 mg CY per kg leads to faster tumor regression than that obtained with 15 mg CY per kg.

Evaluation of the Direct Tumoricidal Effect of a Low or a High Dose of CY for Tumor Cells in the Primary Tumor Nodule. Mice bearing a large tumor (21 mm on Day 12) were given a single i.p. injection of either 15 or 200 mg CY per kg. On various days after CY therapy, the tumor nodule was excised, a single-cell suspension was prepared, and cells obtained from each mouse were injected s.c. into 3 normal BALB/c mice. The normal mice were then monitored for 60 days for the appearance of lethal tumors (Chart 2). When cells were obtained from a tumor nodule 1, 2, or even 3 days after CY therapy with 15 mg/kg, at a time when CY and its active metabolites had essentially been cleared from the circulation (7), the cells established tumors in a significant number of new recipients (10 of 18, 4 of 9, and 3 of 9, respectively). In contrast, when cells were obtained from a tumor nodule even 1 day after CY therapy with 200 mg/kg, the cells failed to establish tumors in any of the new recipients (0 of 18). Thus, the curative effectiveness of 15 mg CY per kg is not due solely to its tumoricidal effect [in confirmation of previous observations (21)], whereas the curative effectiveness of 200 mg CY per kg appears to be primarily due to the tumoricidal effect of the drug.

Effect of ATS on the Curative Effectiveness of a Low or a High Dose of CY for Mice Bearing a Large Tumor. Experiments were performed to determine whether participation of antitumor immunity is required for CY-induced tumor eradication with a low or a high dose of drug. Mice bearing a 21-mm tumor were given a single i.p. injection of either 15 or 200 mg CY per kg. Subsequently, one-half of the mice in each group were treated with ATS on Days 2, 4, and 7 after CY therapy (Chart 3). ATS abolished the curative effectiveness of 15 mg CY per kg, whereas ATS did not reduce the curative effectiveness of 200 mg CY per kg.
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15mg/kg CY

200mg/kg CY

Days Post CY Therapy When Cells Were Obtained From Tumor Nodules

Chart 2. The ability of cells obtained from the tumor nodule of tumor-bearing mice treated with either 15 or 200 mg CY per kg to establish lethal tumors upon administration into new normal recipients. Numbers above bars, number of mice with tumors per number of mice inoculated.

Effect of CY Therapy with a Low or a High Dose on the Antitumor Immune Potential of Spleen Cells from Tumor-bearing Mice. Mice bearing a large tumor were treated with either 15 or 200 mg CY per kg (TuB-15 or TuB-200 mice, respectively). On various days after CY therapy, their spleens were excised, single-cell suspensions were prepared, and the cells were assayed for their antitumor immune potential. This was done by determining the ability of the spleen cells to mount an in vitro antitumor cytotoxic response following in vitro immunization by cocultivation with mitomycin C-treated stimulator tumor cells (Chart 4). In vitro-immunized spleen cells from untreated mice bearing a large tumor exhibited a lower level of antitumor cytotoxicity than did in vitro-immunized spleen cells from normal mice [in confirmation of our previous observation (27, 28)]. As early as 1 day after therapy of tumor-bearing mice with 15 mg CY per kg, their spleen cells exhibited an augmented level of antitumor immune potential, which was further augmented by Day 3 and was maintained at about the same high level for at least 12 days. In contrast, following therapy of tumor-bearing mice with 200 mg CY per kg, their spleen cells exhibited a drastically reduced level of antitumor immune potential which recovered gradually, exceeding the antitumor immune potential of spleen cells from untreated tumor-bearing mice by Day 8. However, at all times after CY therapy, the antitumor immune potential of spleen cells from TuB-15 mice was greater than that of spleen cells of TuB-200 mice. Moreover, upon in vitro immunization, spleen cells from TuB-15 behaved like immune spleen cells, generating higher levels of antitumor cytotoxic activity than did in vitro immunized spleen cells from normal mice. Thus, spleen cells from TuB-15 mice exhibited, following in vitro immunization, much higher levels of antitumor cytotoxicity than do spleen cells from TuB-200 mice.

Ability of Spleen Cells Obtained from Tumor-bearing Mice Treated with a Low or a High dose of CY to Confer Systemic Antitumor Immunity. Mice bearing a large tumor were treated with either 15 or 200 mg CY per kg, and 6 days later, when their tumors were regressing, the spleens were removed for use in adoptive transfer experiments. The effectiveness of the spleen cells in conferring systemic antitumor immunity was assessed by the ability of the cells to aid in the cure of mice bearing a nonpalpable tumor in conjunction with a subcurative dose of CY. Mice bearing a nonpalpable Day 4 tumor were given a single i.p. injection of a subcurative dose of CY (15 mg/kg), and 1 day later, the mice received an i.v. injection of $5 \times 10^6$ spleen cells (Chart 5). All but one of the mice bearing a nonpalpable tumor and treated with CY alone or CY in conjunction with normal spleen cells died. On the other hand, almost all (90%) of the mice treated with CY in conjunction with spleen cells from TuB-15 mice were cured. In contrast, none of the mice treated with CY in conjunction with spleen cells from TuB-200 mice was cured.

Effect of CY Therapy with a Low or a High Dose on the Antitumor Immune Potential of Spleen Cells from Tumor-bearing Mice. Mice bearing a 23-mm tumor were treated with either 15 or 200 mg CY per kg (TuB-15 or TuB-200 mice, respectively). On various days after CY therapy, their spleens were excised, single-cell suspensions were prepared, and the cells were assayed for their antitumor immune potential. This was done by determining the ability of the spleen cells to mount an in vitro antitumor cytotoxic response following in vitro immunization by cocultivation with mitomycin C-treated stimulator tumor cells (Chart 4). In vitro-immunized spleen cells from untreated mice bearing a large tumor exhibited a lower level of antitumor cytotoxicity than did in vitro-immunized spleen cells from normal mice [in confirmation of our previous observation (27, 28)]. As early as 1 day after therapy of tumor-bearing mice with 15 mg CY per kg, their spleen cells exhibited an augmented level of antitumor immune potential, which was further augmented by Day 3 and was maintained at about the same high level for at least 12 days. In contrast, following therapy of tumor-bearing mice with 200 mg CY per kg, their spleen cells exhibited a drastically reduced level of antitumor immune potential which recovered gradually, exceeding the antitumor immune potential of spleen cells from untreated tumor-bearing mice by Day 8. However, at all times after CY therapy, the antitumor immune potential of spleen cells from TuB-15 mice was greater than that of spleen cells of TuB-200 mice. Moreover, upon in vitro immunization, spleen cells from TuB-15 behaved like immune spleen cells, generating higher levels of antitumor cytotoxic activity than did in vitro immunized spleen cells from normal mice. Thus, spleen cells from TuB-15 mice exhibited, following in vitro immunization, much higher levels of antitumor cytotoxicity than do spleen cells from TuB-200 mice.

Ability of Spleen Cells Obtained from Tumor-bearing Mice Treated with a Low or a High dose of CY to Confer Systemic Antitumor Immunity. Mice bearing a large tumor were treated with either 15 or 200 mg CY per kg, and 6 days later, when their tumors were regressing, the spleens were removed for use in adoptive transfer experiments. The effectiveness of the spleen cells in conferring systemic antitumor immunity was assessed by the ability of the cells to aid in the cure of mice bearing a nonpalpable tumor in conjunction with a subcurative dose of CY. Mice bearing a nonpalpable Day 4 tumor were given a single i.p. injection of a subcurative dose of CY (15 mg/kg), and 1 day later, the mice received an i.v. injection of $5 \times 10^6$ spleen cells (Chart 5). All but one of the mice bearing a nonpalpable tumor and treated with CY alone or CY in conjunction with normal spleen cells died. On the other hand, almost all (90%) of the mice treated with CY in conjunction with spleen cells from TuB-15 mice were cured. In contrast, none of the mice treated with CY in conjunction with spleen cells from TuB-200 mice was cured.
Low- versus High-Dose Tumor Chemotherapy

Chart 5. The ability of spleen cells obtained from tumor-bearing mice treated with 15 or 200 mg CY per kg to confer systemic antitumor immunity. Mice bearing a nonpalpable tumor were treated with a subcurative dose of CY (15 mg/kg) alone or in conjunction with spleen cells from normal mice or tumor-bearing mice treated with either 15 (TuB-15) or 200 (TuB-200) mg CY per kg 6 days earlier. Numbers above bars, number of mice cured per number of mice treated.

Thus, whereas spleen cells from TuB-15 mice are effective in conferring systemic antitumor immunity, spleen cells from TuB-200 mice do not do so effectively.

Kinetics of Development of the Ability to Confer Systemic Antitumor Immunity in Spleens of TuB-15 Mice. Experiments were performed to determine how soon after CY therapy the spleens of mice bearing a 22-mm tumor are able to confer systemic antitumor immunity to mice bearing a nonpalpable tumor, thereby resulting in their cure in conjunction with a subcurative dose of CY (15 mg/kg). Mice bearing a nonpalpable tumor were treated with CY alone or in conjunction with 50 \times 10^6 spleen cells from untreated tumor-bearing mice or tumor-bearing mice treated with CY (15 mg/kg) 1, 3, 6, or 8 days earlier (Chart 6). Spleen cells obtained from either untreated tumor-bearing mice or tumor-bearing mice treated with CY (15 mg/kg) up to 3 days earlier were unable to confer systemic antitumor immunity. However, spleen cells obtained 6 or 8 days after chemotherapy were effective in conferring systemic antitumor immunity and, in conjunction with a subcurative dose of CY, resulted in the cure of most mice (93 and 80%, respectively). Thus, by Day 6 after CY therapy with 15 mg/kg, cells capable of conferring systemic antitumor immunity are present in the spleen of tumor-bearing mice.

Effect of Spleen Cells from TuB-200 Mice on the Ability of Spleen Cells from TuB-15 Mice to Confer Systemic Antitumor Immunity. Experiments were performed to determine whether the inability of spleen cells from mice bearing a large tumor and treated with 200 mg CY per kg to confer systemic antitumor immunity is due to drug-induced suppressor elements. This was done by evaluating whether the spleen cells from mice bearing a large tumor when treated with 200 mg CY per kg 6 days earlier could suppress the ability of spleen cells from tumor-bearing mice treated with 15 mg CY per kg 6 days earlier to confer systemic antitumor immunity. Mice bearing a nonpalpable tumor were treated with a subcurative dose of CY alone or in conjunction with (a) 50 \times 10^6 spleen cells from TuB-15 mice, (b) 50 \times 10^6 spleen cells from TuB-200 mice, or (c) a mixture of 50 \times 10^6 spleen cells from TuB-15 mice plus 50 \times 10^6 spleen cells from TuB-200 mice (Chart 7). Spleen cells from mice bearing a large tumor and treated with 15 mg CY per kg but not 200 mg CY per kg were able to confer systemic antitumor immunity to mice...
bearing a nonpalpable tumor and cured most of them (87%) in conjunction with a subcurative dose of CY. The ability of the spleen cells from TuB-15 mice to confer systemic antitumor immunity was not reduced when mixed with an equal number of spleen cells from TuB-200 mice, and upon administration to mice bearing a nonpalpable tumor in conjunction with a subcurative dose of CY, it cured most mice bearing a nonpalpable tumor (87%). Thus, treatment of mice bearing a large tumor with 200 mg CY per kg does not lead to the appearance of suppressor elements that interfere with the conferral of systemic antitumor immunity.

Ability of Mice Bearing a Large Tumor and Treated with a Low or a High Dose of CY to Reject a Lethal Tumor Challenge. Mice bearing a 22-mm tumor were treated with either 15 or 200 mg CY per kg and challenged 1, 6, or 31 days later with 1 × 10⁶ viable tumor cells, which is about 300-fold greater than the minimal lethal dose for 100% of normal mice (Chart 8). Mice bearing a large tumor and treated with 15 mg CY per kg were able to reject a lethal challenge given even 1 day after CY therapy, and they retained this ability for at least 31 days. In contrast, mice bearing a large tumor and treated with 200 mg CY per kg were unable to reject such a challenge given at the same time intervals after CY therapy. Thus, although a dose of 200 mg CY per kg cures most mice bearing a large MOPC-315 tumor, it does not result in antitumor immunity, in contrast to mice cured with 15 mg CY per kg which exhibit long-lasting potent antitumor immunity.

Ability of TuB-200 Mice to Eradicate a Second Nonpalpable Tumor following Treatment with a Subcurative Dose of CY with or without Adoptively Transferred Immune Cells. The curative effectiveness of 15 mg CY per kg alone or in conjunction with immune spleen cells for a nonpalpable Day 4 tumor established in TuB-200 mice was compared to its effectiveness for a nonpalpable Day 4 tumor established in normal mice (Chart 9). Most of the nonpalpable Day 4 tumors established in normal mice were not eradicated by CY therapy with 15 mg/kg [in confirmation of our previous observations (20, 29)]. Similarly, this dose of CY did not eradicate most of the nonpalpable tumors established in TuB-200 mice. However, this low dose of CY in conjunction with adoptively transferred immune spleen cells eradicated a substantial number of tumors established in normal mice. The effectiveness of chemoimmunotherapy for the eradication of a nonpalpable tumor established in TuB-200 mice was slightly reduced, compared to its effectiveness for a nonpalpable tumor established in normal mice, when 5 × 10⁷ immune cells were transferred (33% versus 53%, respectively). The difference in the effectiveness of the therapy was magnified when lower numbers of immune cells were transferred (13 versus 47% with 2.5 × 10⁶ or 0 versus 40% with 1.25 × 10⁶ immune spleen cells). Thus, treatment of tumor-bearing mice with a dose of 200 mg CY per kg reduces the curative effectiveness of chemoimmunotherapy for a subsequent tumor challenge.

**DISCUSSION**

We have shown previously that mice bearing a large MOPC-315 tumor could be cured with a single i.p. injection of 15 to 200 mg CY per kg (20). Here, we compared tumor cure induced by a low dose of CY (15 mg/kg) with that induced by a high dose of CY (200 mg/kg). Tumor regression occurred faster following CY therapy with the high dose of CY than following therapy with the low dose. Tumor regression induced with 200 mg CY per kg was due solely to tumoricidal effect of the drug, whereas the regression induced by 15 mg CY per kg required cooperation between the toxic effects of the drug and host antitumor immunity. CY modulated the antitumor immunity of tumor-bearing mice such that spleen cells from mice treated with the low dose of CY exhibited augmented antitumor potential, whereas spleen cells from mice treated with the high dose of CY exhibited suppressed antitumor potential. Tumor-bearing mice cured following therapy with 15 mg CY per kg exhibited long-lasting potent antitumor immunity as was evident from their ability to reject a lethal tumor challenge. In contrast, although 200 mg CY per kg cured most mice bearing a large tumor, it did not result in antitumor immunity, but it actually reduced the effectiveness...
of chemoimmunotherapy for a second tumor challenge.

The studies on the kinetics of tumor regression demonstrate a decrease in tumor diameter as early as 1 day following CY therapy with 200 mg/kg but not within the first 2 days following CY therapy with 15 mg/kg. Although it is tempting to conclude from these data that reduction in tumor load was first seen at a shorter time interval following CY therapy with 200 mg/kg than following CY therapy with 15 mg/kg, such a conclusion is not justified. At any time, a tumor nodule may consist of tumor cells and host cells, necrotic material, and intracellular fluid. Changes in the size of the tumor nodule may reflect changes in any or all of these parameters. On the other hand, no change in tumor size does not necessarily mean that no change in tumor site composition occurred. Although it is not clear from these data whether a decrease in tumor load occurred sooner following CY therapy with 200 mg/kg than with 15 mg/kg, the data demonstrate clearly that complete rejection of the primary tumor occurred faster following CY therapy with 200 mg/kg than following CY therapy with 15 mg/kg.

Although regression of a large MOPC-315 tumor can be induced with 15 or 200 mg CY per kg, the mechanism of CY-induced tumor eradication is quite different with each dosage. Viable tumor cells were not detected in the tumor nodule of mice even 1 day after treatment with 200 mg CY per kg, whereas viable tumor cells were detected in the tumor nodule of a substantial portion of the mice even 3 days after therapy with 15 mg CY per kg. Since CY and its active metabolites are cleared from the circulation within 4 to 6 hr after CY administration (7), our results suggest that CY-induced tumor eradication with the high dose of drug is due to the tumoricidal effects of the drug, while the tumoricidal effects of CY alone cannot account for tumor eradication with 15 mg/kg. This conclusion is substantiated by our results demonstrating that the curative effect of 200 mg CY per kg did not require the participation of T-cell-dependent antitumor immunity, whereas the curative effect of 15 mg CY per kg did require the participation of T-cell-dependent antitumor immunity.

Spleen cells from mice bearing a large MOPC-315 tumor generated lower levels of antitumor cytotoxicity following in vitro immunization than did in vitro-immunized spleen cells from normal mice [in confirmation of our previous observations (27, 28)]. Spleen cells obtained from CY-treated tumor-bearing mice within 3 days after CY therapy with 15 mg/kg, but not with 200 mg/kg, exhibited augmented ability to mount cytotoxic antitumor responses following in vitro immunization. The reduced levels of antitumor cytotoxicity exhibited, upon in vitro immunization, by spleen cells derived from tumor-bearing mice treated with 200 mg CY per kg were not due to use of inappropriate immunization conditions. In addition, although CY has been shown, under certain conditions, to induce suppressor T-cells (5), it is unlikely that they are responsible for the reduced antitumor immune potential of spleen cells from tumor-bearing mice treated with 200 mg CY per kg. This is illustrated by the failure of such spleen cells to suppress the ability of spleen cells derived from tumor-bearing mice treated with 15 mg/kg to confer systemic antitumor immunity. The most likely explanation for the reduced antitumor immune potential of spleen cells from tumor-bearing mice treated with 200 mg CY per kg is drug-induced elimination or inactivation of cells involved in the generation and/or expression of antitumor immunity.

Other investigators (16, 24–26, 32) have shown that treatment of normal recipients with a high dose of CY suppresses the ability of their spleen cells to mount antitumor cytotoxic responses following immunization with allogeneic tumor cells. This was not due to elimination of precursors of cytotoxic T-lymphocytes but rather to elimination of helper cells required for the development of the cytotoxic T-cells (24). The ability to generate cytotoxic lymphocytes was restored to the spleens of mice treated with a high dose of CY by addition of Ly 1+ T-lymphocytes (helper T-cells) (25) or by addition of supernatants of mixed leukocyte cultures which contained helper factors (26). It has to be determined whether, in the MOPC-315 tumor system, too, the reduced ability of spleen cells from tumor-bearing mice to mount an antitumor cytotoxic response following in vitro immunization is due to the elimination of helper T-cells.

Although the ability to exhibit augmented levels of antitumor cytotoxicity following in vitro immunization developed in the spleens of tumor-bearing mice by Day 3 after CY therapy with 15 mg/kg, the ability to confer systemic antitumor immunity developed at a later time (by Day 6). The apparent earlier appearance of in vitro antitumor cytotoxic potential might be a consequence of the assay method used for its evaluation. In the in vitro immunization process, the spleen cells are cultured for 5 days in the presence of stimulator tumor cells prior to their assay for antitumor cytotoxicity. This time period might be enough for the in vitro maturation of precytotoxic cells into cytotoxic cells. On the other hand, preliminary results (data not shown) have indicated that, in the in vivo assay, a delay in transferring immune cells after CY therapy drastically reduced their therapeutic effectiveness. Thus, the in vivo assay appears to provide information on the therapeutic effectiveness of the cells at the time of adoptive transfer or shortly thereafter, whereas the in vitro assay provides information regarding the cytotoxic activity of the cells after 5 days in culture. Therefore, it is important to determine whether an increase in the ability of spleen cells, obtained on Day 3 after CY therapy with 15 mg/kg, to confer systemic antitumor immunity can be accomplished by in vitro immunization of the spleen cells prior to their use in adoptive transfer experiments. Alternatively, it is possible that different cell type(s) or different numbers of cells of the same type mediate the in vivo and the in vitro activities. Recently, it has been shown that the predominant cell type responsible for the therapeutic effectiveness of adoptively transferred immune cells is a Ly 1+ T-cell that is not cytotoxic at the time of therapy (19). However, it is not known at present if the Ly 1+ cells promote tumor eradication by providing help to antibody responses, acting as initiators of delayed-type hypersensitivity responses or amplifying cell-mediated cytotoxic responses. Although Ly 1+ cells might also amplify the in vitro generation of antitumor cytotoxic responses (25), lower numbers or a different subset of Ly 1+ T-cells might be required than those required to confer systemic antitumor immunity.

Combined chemoimmunotherapy was less effective for the eradication of a Day 4 nonpalpable tumor established in TuB-200 mice than for a Day 4 nonpalpable tumor established in normal mice. The decrease in the effectiveness of chemoimmunotherapy for the Day 4 challenge was due to previous treatment of the mice with a high dose of CY, which resulted in either a decrease in the therapeutic effectiveness of the drug and/or a decrease in the therapeutic effectiveness of the adoptively trans-

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*M. B. Mokyr and S. Dray, unpublished observations.*
ferred immune cells. The decrease in the therapeutic effectiveness of the drug might have been due, in part, to a decrease in the conversion of CY into its active metabolites. On the other hand, the decrease in the therapeutic effectiveness of the adoptively transferred immune cells might have been due, in part, to the elimination of host factors required for conferral of systemic antitumor immunity and/or to the induction of suppressor elements (5) that inhibit the activity of the adoptively transferred cells. It has been suggested that adoptively transferred immune cells mediate their protective activity by recruitment of host cells (37). The need for an intact recipient antitumor immune potential for successful chemoimmunotherapy of tumor-bearing mice at an early stage of tumor growth has been demonstrated in the FBL-3 tumor system (18). Combined chemotherapy and adoptive immunotherapy for mice bearing a Day 5 FBL-3 tumor did not eliminate all metastatic tumor cells, and there was a transient period of regrowth of metastatic tumor cells in the spleen; nevertheless, the metastases were later eradicated, and the mice were cured. However, when the recipients of chemotherapy were immunosuppressed by treatment with ATS, the chemoimmunotherapy did not eradicate the metastatic tumor cells, resulting in recurrence of lethal tumors.

Recently, it has been shown that, in patients with advanced cancers, treatment with CY prior to immunization with keyhole limpet hemocyanin or 1-chloro-2,4-dinitrobenzene resulted in the development of delayed-type hypersensitivity responses to these antigens in otherwise unreactive patients (2, 3, 22). Reversal of T-cell anergy was seen in these studies even though the conventional dose of CY used (100 mg/kg) led to a drastic reduction in the number of peripheral blood lymphocytes. The authors ascribed the CY-induced augmentation of delayed-type hypersensitivity responses to selective impairment of suppressor functions. However, they emphasized that it is possible that, at this high dose of CY, some of the immunopotentiating effects of the drug are masked by the immunosuppressive effects, and a lower dose of CY might be more effective in augmenting T-cell-mediated immune responses (3). Our results support this hypothesis in an animal tumor model. In the MOPC-315 tumor system, too, a gradual recovery of antitumor immune potential of spleen cells from tumor-bearing mice treated with the high dose of CY occurred. By Day 15 following CY therapy, such spleen cells generated higher levels of antitumor cytotoxicity upon in vitro immunization than did in vitro-immunized spleen cells from normal mice. However, although some augmentation in antitumor immune potential of spleen cells from tumor-bearing mice was seen following CY therapy with 200 mg/kg, it occurred more slowly and was weaker than the augmentation obtained following CY therapy with 15 mg/kg.

There is an increasing awareness among investigators that the efficacy of oncotherapeutic regimens is influenced by the immune status of the host (10, 23, 30, 33). Accordingly, patients with normal immune profiles have been reported to respond more favorably to therapy than patients with suppressed immune responsiveness (11, 12). A common practice in clinical oncology is to treat patients with the maximal tolerable dose of drug (4) so as to maximize the tumoricidal activity of the drug (36). However, such therapeutic regimens are often immunosuppressive. Moreover, patients treated “successfully” with chemotherapy are subject to a higher risk of developing a different type of cancer (13, 17). These observations in humans as well as our observations in the murine plasmacytoma model suggest that therapy with a high dose of drug might not always be superior to therapy with a low dose of drug. This might occur for a tumor which is sufficiently immunogenic to induce an appreciable level of antitumor immunity that can cooperate effectively with the low dose of drug in tumor eradication.

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REFERENCES


Some Advantages of Curing Mice Bearing a Large Subcutaneous MOPC-315 Tumor with a Low Dose Rather Than a High Dose of Cyclophosphamide

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