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Increase in the Effectiveness of Melphalan Therapy with Progression of MOPC-315 Plasmacytoma Tumor Growth

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Summary. *Following inoculation with 1×10^6 MOPC-315 tumor cells, a single injection of a very low dose of melphalan (L-PAM, L-phenylalanine mustard), 0.75 mg/kg, cured most of the mice bearing a day 11 large primary tumor (20 mm) and metastases, but failed to cure mice bearing a day 4 nonpalpable tumor. Treatment of mice bearing a nonpalpable tumor with the very low dose of drug compromised the ability of the mice to respond effectively to the same low dose of drug when the tumor became large (day 12). However, a nonpalpable tumor could be eradicated by treatment of tumor bearers with a low dose of L-PAM, if it was present concomitantly with a large tumor on the contralateral side. A high dose of L-PAM, 15 mg/kg, cured mice bearing either a nonpalpable or a large tumor. The eradication of the tumor induced by the high dose of L-PAM appeared to be due solely to the tumoricidal effect of the drug. On the other hand, the eradication of the tumor by the low dose of L-PAM also required the participation of antitumor immunity of the host, since subsequent injection of antithymocyte serum abrogated the curative effect of the drug in most mice. Mice cured by a high dose of L-PAM were not resistant to subsequent lethal tumor challenge. In contrast, mice cured by the low dose of L-PAM were able to reject a tumor challenge of 300 times the minimal lethal tumor dose. The results obtained with L-PAM therapy are similar to the results that we had previously reported with cyclophosphamide therapy. Thus, the timing of therapy with a low dose of drug for mice bearing a MOPC-315 tumor is critical for successful therapy. Moreover, the selection of a low dose rather than a high dose of drug to eradicate a large tumor offers the advantage that it results in long-lasting potent antitumor immunity as a consequence of the participation of host antitumor immunity in the eradication of the tumor.*

Introduction

Cancer chemotherapy can be influenced by antitumor immunity. The effectiveness of chemotherapy was decreased when tumor-bearing animals were immunosuppressed with antithymocyte serum [11, 14, 26], x-irradiation [16, 26, 31], or high doses of drug [17, 31]. The effectiveness of chemotherapy was increased in the presence of antitumor immunity developed by preimmunizing the tumor-bearing animals [5, 17] or by adoptively transferring immunity with lymphoid cells [7, 11, 12, 18, 21, 23]. Several mechanisms have been suggested for cooperation between chemotherapy and host antitumor immunity. Accordingly, the drug might do the following: (a) reduce the tumor burden to a level whereby existent host antitumor immunity can eliminate residual tumor cells [5]; (b) slow tumor growth long enough to allow the development of potent host antitumor immunity [5]; (c) render residual tumor cells more immunogenic, thereby providing a superior stimulation for the development of host antitumor immunity [2, 8, 9]; (d) render residual tumor cells more susceptible to immune lysis [4]; or (e) eliminate suppressor cells [14, 23], thereby allowing the generation and/or expression of potent host antitumor immunity [14].

We have recently demonstrated the importance of the timing and of the dose of cyclophosphamide (CY) administered to MOPC-315 tumor-bearing mice for the outcome of the therapy [6, 13, 18]. Accordingly, the curative effect of CY increased with progression of tumor growth, in such a way that a low dose (15 mg/kg) which rarely cured mice bearing a nonpalpable (day 4) tumor was curative for most mice bearing a large tumor (20–25 mm, day 12–16). The ineffectiveness of a low dose of CY at an early stage of tumor growth was due to insufficient levels of antitumor immunity [6, 18, 21]. On the other hand, the effectiveness of the low dose of CY at an advanced stage of tumor growth was due to cooperation between the drug's cytotoxic effect and the host's antitumor immunity [14]. The importance of the timing of chemotherapy was further illustrated by the inability to cure mice bearing a large tumor with a low dose of CY if the mice were previously treated with a low dose of CY when they had a nonpalpable tumor [13]. Finally, mice cured by the low dose of CY, 15 mg/kg, were resistant to subsequent tumor challenge, whereas mice cured by a high dose of CY, 200 mg/kg, were not [6, 18].

The purpose of this study was to ascertain whether the general concepts that emerged from the work with CY are applicable to another drug. Melphalan (L-PAM) was selected for this study, since it is an alkylating agent which, unlike CY, does not depend on hepatic mixed function oxidases to exert its cytotoxic activity [25, 29]. In addition, L-PAM is widely used in clinical oncology, particularly in the treatment of multiple myeloma [30]. Here, we present an evaluation of the importance of the timing and of the dose of L-PAM administered for the therapy of mice bearing the MOPC-315 plasmacytoma.

Materials and Methods

Tumors. The MOPC-315 plasmacytoma was maintained by serial SC inoculations into the lower flank of syngeneic

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BALB/c mice (8–12 weeks old; Goodwin Cancer Research Institute, Plantation, FL, USA). Routinely, mice were inoculated with 1×10^6 viable MOPC-315 tumor cells, a dose which leads invariably to progessively growing tumors that kill the mice in a mean time of 20 ± 1 days (SE). Single-cell suspensions were prepared by mechanical disruption of tumor pieces in plain RPMI 1640 medium (Grand Island Biological Company, Grand Island, NY, USA). The number of cells was determined by the use of a Coulter counter. The viability of tumor cells was determined by exclusion of trypan blue (0.4%) and exceeded 85%.

Spleen Cell Suspensions. Single-cell suspensions were prepared by mechanical disruption in Eagle's minimal essential medium (GIBCO, Grand Island, NY, USA) and counted in a Coulter counter. The viability determined by trypan blue dye exclusion always exceeded 95%. The presence of tumor cells in the spleen was determined by injection of 5×10^7 cells in 0.5 ml to mice SC and looking for the appearance of tumors during a 60-day observation period.

Chemotherapy. Melphalan (L-PAM) powder (Burroughs-Wellcome Co., Research Triangle Park, NC, USA) was used. A concentrated solution of 10 mg/ml or 20 mg/ml was prepared immediately before injection in a solvent mixture composed of one volume acid alcohol (5 : 1 of 95% ethyl alcohol : 2 *N* HCl) and one volume of propylene gylcol buffer pH 7.2, [K_2HPO_4 2% w/v, propylene glycol (Sigma Co., St Louis, MO, USA) 45 ml and distilled water to give 100 ml final volume]. Further dilutions were made in Dulbecco's phosphate-buffered saline (GIBCO, Grand Island, NY, USA) and used immediately to minimize hydrolysis. All doses of L-PAM were given as a single IP injection of 0.5 ml unless indicated otherwise. Tumors were measured three times weekly with a vernier caliper. Mice receiving L-PAM which remained tumor-free 60 days after the tumor inoculation were considered to be cured. Treated mice were challenged 30 or 60 days after the first tumor inoculation with viable MOPC-315 tumor cells SC on the contralateral flank, and were observed again for appearance of tumors for an additional period of 60 days.

Rabbit Anti-Mouse Thymocyte Serum. Rabbit anti-mouse thymocyte serum (Microbiological Associates, Walkersville, MD) was stored at −20° C prior to use. Mice were given three IP injections of 0.25 ml each on days 2, 4, and 6 post L-PAM therapy. This protocol of anti-thymocyte serum treatment was shown to cause a 93% reduction in the number of Thy 1.2+ cells in the spleen and to virtually abolish the ability of spleen cells to proliferate in response to a T cell mitogen, phytohemagglutinin (94% reduction), and to generate antitumor immunity (100% reduction). This protocol, however, did not reduce the ability of the spleen cells to proliferate in response to a B cell mitogen, lipopolysaccharide.

Results

Effectiveness of Therapy with a Low Dose of L-PAM for Mice Bearing Various Sizes of MOPC-315 Tumors

Mice were inoculated SC with 1×10^6 viable tumor cells, a dose which is about 300 times the minimal lethal tumor dose and leads to the death of all mice in 20 ± 1 days. On various days post tumor inoculation, different groups of mice were given a single IP injection of L-PAM, 0.75 mg/kg, when the tumor was nonpalpable (day 4), small (6 mm; day 7), large (20 mm; day 11), or very large (29 mm; day 14) (Fig. 1). The effectiveness of chemotherapy increased with the progression of tumor growth from day 4 (0% cure) until day 11 (95% cure). However, when the tumor became very large (day 14) the chemotherapy had little effect (20% cure).

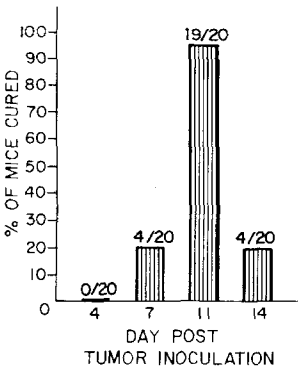

Fig. 1. Curative effectiveness of a single low dose of L-PAM for mice bearing a tumor at different stages during the progression of tumor growth. After SC inoculation of 1×10^6 MOPC-315 tumor cells, a single dose of L-PAM, 0.75 mg/kg, was given IP to different groups of mice on: day 4 (nonpalpable tumor); day 7 (small, palpable tumor; 5.3 mm ± 0.6 SE); day 11 (large tumor; 21.0 mm ± 0.2 SE); or day 14 (very large tumor; 29.6 mm ± 0.3 SE). Ratios above the *bars* represent the number of cured mice/total. The figure represents combined data of two experiments

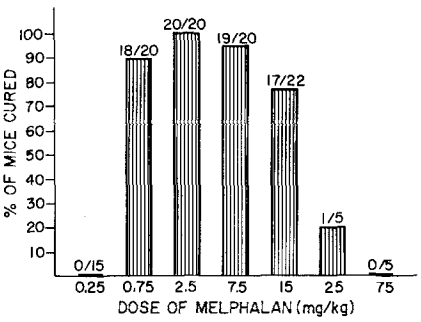

Fig. 2. Dose response to L-PAM of mice bearing a large tumor (day 11, 20 mm). Ratios above the *bars* represent the number of cured mice/total. The figure represents combined data of three experiments except for the doses of 25 and 75 mg/kg

Dose Response to L-PAM of Mice Bearing Large Tumors

Mice bearing a large (20 mm, day 11) tumor were given single injections of L-PAM in doses ranging from 0.25 to 75 mg/kg (Fig. 2). With the dose of 0.25 mg/kg, no mice were cured. In the dose range of 0.75–15 mg/kg, most mice were cured. When larger doses of L-PAM (25 or 75 mg/kg) were injected the tumors showed marked regression but the mice died within a few days due to general toxicity to the drug.

The kinetics of tumor regression was determined for mice receiving an L-PAM dose of 0.75 mg/kg (Fig. 3). No change in tumor size was observed within the first 2 days post therapy, the tumor size decreased significantly by day 3 post therapy, and the tumor regressed completely by day 10.

Dose Response to L-PAM of Mice Bearing Nonpalpable Tumors

Since a very low dose of L-PAM (0.75 mg/kg) did not cure mice bearing a nonpalpable (day 4) tumor, experiments were done

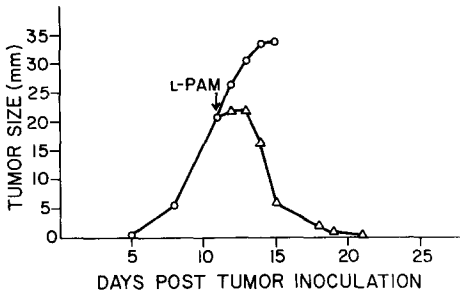

Fig. 3. Kinetics of tumor growth in untreated mice (○) and in mice treated with L-PAM when they had a large tumor. Mice were inoculated with 1×10^6 MOPC-315 tumor cells. On day 11, when the tumor was 20 mm, half the mice (△) received a single IP injection of L-PAM, 0.75 mg/kg. Each *point* represents the mean tumor diameter (mm) of 10 mice

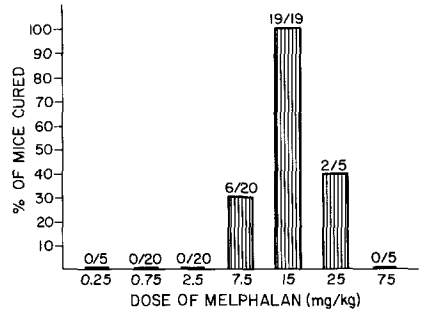

Fig. 4. Dose response to L-PAM of mice bearing a nonpalpable tumor (day 4). Ratios above the *bars* represent the number of cured mice/total. The figure represents combined data of two experiments except for the doses of 0.25, 25, and 75 mg/kg

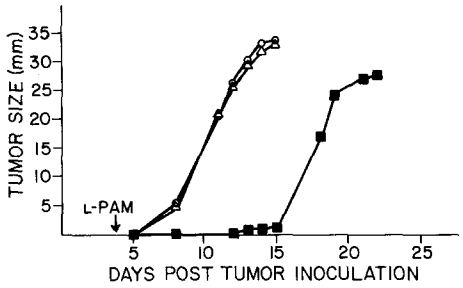

Fig. 5. Kinetics of tumor growth in untreated mice and in mice treated with L-PAM when they had a nonpalpable tumor (day 4). Mice were inoculated with 1×10^6 MOPC-315 tumor cells. On day 4 the mice were divided into three groups (10 mice in each group): untreated (○); treated with L-PAM, 0.75 mg/kg (△); and treated with L-PAM, 7.5 mg/kg (■)

to determine whether mice bearing a day 4 tumor could be cured by a higher dose of the drug (Fig. 4). When low doses of L-PAM, up to 2.5 mg/kg, were administered no mice were cured. A few mice were cured by a dose of 7.5 mg/kg, but a very high dose of 15 mg/kg was required to prevent development of tumors in all the mice. Injection of still higher doses of L-PAM (25 or 75 mg/kg) led to the death of most of the mice within 2–5 days due to the general toxicity of the drug.

Since the 0.75 mg/kg or 7.5 mg/kg doses of L-PAM cured most mice bearing large tumors but not mice bearing nonpalpable tumors, experiments were performed to determine whether the low dose of drug can at least delay the progressive growth of the nonpalpable tumor (Fig. 5). Mice treated on day 4 with a noncurative 0.75 mg/kg dose of L-PAM developed tumors at approximately the same time as the untreated controls and the kinetics of tumor growth was essentially the same, i.e., the mean survival was 19 ± 1 days for the control group and 20 ± 1 days for the L-PAM-treated group. However, for the 14 mice not cured by the 7.5 mg/kg dose of L-PAM, the time of tumor appearance was delayed by about 10 days but once the tumor appeared, the kinetics of tumor growth was similar to that in mice treated with the lower doses of drug, i.e., the mean survival time was extended to 32 ± 3 days.

Table 1. Effectiveness of a low dose of L-PAM, 0.75 mg/kg, for therapy of mice bearing a large MOPC-315 tumor not cured by previous therapy when they had a nonpalpable tumor

Experimental groups	Effect of therapy[a]	
	Cured (%)	No. of cured/ total
Untreated tumor bearers	0	0/20
Treated large-tumor bearers[b]	100	20/20
Treated nonpalpable-tumor bearers[c]	0	0/20
Double-Treated tumor bearers[d]	30	6/20

[a] Combined results of 2 experiments
[b] Mice treated on day 11 when tumors were 20.8 mm ± 0.5 (SE)
[c] Mice treated on day 4 after tumor inoculation
[d] Mice treated with L-PAM on day 4 after tumor inoculation and again when the tumor reached 20.8 ± 0.5 (SE) (day 11)

Effectiveness of a Low Dose of L-PAM for the Cure of Mice Bearing a Large Tumor after Treatment with a Noncurative Low Dose of L-PAM when the Tumor was Nonpalpable

Since mice bearing a nonpalpable tumor were not cured by a 0.75 mg/kg dose of L-PAM, experiments were performed to determine whether these mice could be cured by a second injection of the same low dose of L-PAM when the tumor had become large (day 11, 21 mm) (Table 1). As expected, a single dose of L-PAM, 0.75 mg/kg, did not cure any of the mice when given on day 4 but did cure almost all of the mice when given on day 11 post tumor inoculation (Figs. 1, 2, and 4). However, only 30% of the mice bearing a large tumor were cured by the same low dose of drug if the mice had been previously treated on day 4 with L-PAM. Thus, a low dose of L-PAM given at the stage of a nonpalpable tumor reduced the effectiveness of the drug for curing the mice when the tumor became large.

Effectiveness of a Low Dose of L-PAM for the Eradication of a Nonpalpable Tumor in the Presence of a Large Tumor

Work performed in our laboratory [21] has shown that a low dose of cyclophosphamide can eradicate a nonpalpable MOPC-315 tumor when present concomitantly with a large tumor on the contralateral side. Experiments were done to determine whether a low dose of L-PAM would have a similar effect (Table 2). Groups of mice were inoculated with 1×10^6 MOPC-315 tumor cells on day 0 on the right flank and on day 8 on the left flank. On day 12, when the mice were bearing a nonpalpable tumor concomitantly with a large tumor, they received a single dose of L-PAM, 0.75 mg/kg or 2.5 mg/kg. In 60% of the mice treated with a dose of 0.75 mg/kg and in 80% of the mice treated with a dose of 2.5 mg/kg, both tumors were eradicated. The eradication of the nonpalpable tumor was not

Table 2. Effectiveness of a single IP injection of L-PAM in curing mice bearing a large MOPC-315 tumor on one side and a nonpalpable (day 4) tumor on the contralateral side

Size of tumor at time of chemotherapy	L-PAM therapy	Effect of therapy		
		No. of mice showing tumor at the second site/total	Cured (%)	Cured/ total
Nonpalpable[a]	0.75 mg/kg		0	0/10
Large[b]	0.75 mg/kg		100	10/10
Large and nonpalpable[c]	0.75 mg/kg	6/15	60	9/15
Large and nonpalpable[d]	2.50 mg/kg	0/8	80	8/10
Large and nonpalpable[e]	None	10/10	0	0/10

[a] Mice bearing a nonpalpable (day 4) tumor
[b] Mice bearing a 22.1 ± 0.2 mm (day 12) tumor
[c] Mice bearing a 22.0 ± 0.2 mm (day 12) tumor on one side and a nonpalpable (day 4) tumor on the contralateral side
[d] Mice bearing a 21.9 ± 0.4 mm (day 12) tumor on one side and a nonpalpable (day 4) tumor on the contralateral side
[e] Mice bearing a 21.8 ± 0.3 mm (day 12) tumor on one side and a nonpalpable (day 4) tumor on the contralateral side

Table 3. Presence of viable MOPC-315 tumor cells in the primary tumor nodule and in the spleen of mice following treatment of mice bearing a large tumor with L-PAM[a]

Tumor-bearer donor		Normal recipients	
L-PAM therapy (mg/kg)	Day[b] after L-PAM therapy when cells were obtained	No. of mice developing tumors/total[c] after injection of cells from	
		Tumor nodule[d]	Spleen[e]
–	–	10/10	10/10
0.75	1	10/10	10/10
0.75	3	9/10	3/10
15.00	1	0/8	0/8

[a] L-PAM was injected on day 11 after tumor inoculation when the tumor size reached 20 mm
[b] Cells were obtained from the tumor nodule and the spleen
[c] Combined results of two experiments
[d] 1×10^6 cells per mouse SC
[e] 5×10^7 cells per mouse SC

due to existing concomitant immunity, since the nonpalpable tumor grew progressively in the untreated control mice bearing both a nonpalpable and a large tumor.

The Effect of a Low or a High Dose of L-PAM on the Number of Tumor Cells in the Primary Tumor Nodule and in the Spleen

Mice bearing a large (20 mm, day 11) tumor were given a single IP injection of either a low dose of L-PAM, 0.75 mg/kg, or a high dose of L-PAM, 15 mg/kg. On days 1 or 3 after chemotherapy, the primary SC tumor was excised, the spleen was removed, and single-cell suspensions were prepared.

Table 4. Effect of anti-thymocyte serum (ATS) on eradication of a large MOPC-315 tumor (21.0 ± 0.6 mm) mediated by a low or high dose of L-PAM

Treatment		% Survival	No. surviving/ total[c]
L-PAM dose[a] (mg/kg)	ATS[b]		
None	–	0	0/20
0.75	–	75	15/20
0.75	+	20	4/20
15.00	–	100	6/6
15.00	+	100	8/8

[a] Mice were given a single IP injection of L-PAM
[b] Mice were given 0.25 ml rabbit anti-mouse thymocyte serum IP on days 2, 4, and 6 after L-PAM therapy
[c] The data with a dose of 0.75 mg/kg represent the combined data of two experiments and the data with a dose of 15 mg/kg represent data obtained in one of these experiments

Either 1×10^6 cells from the tumor nodule or 5×10^7 cells from the spleen were injected SC into normal mice. The mice were observed for the appearance of lethal tumors (Table 3). Cells obtained from the tumor nodule and spleen on day 1 or even day 3 after therapy with the low dose of L-PAM, 0.75 mg/kg, established tumors in a significant number of new recipients. Since the half-life of L-PAM is 41 min [10], the L-PAM given at this low dose was essentially cleared from the circulation at the time when viable tumor cells were still present at the primary tumor site and in the spleen of the treated animals. In contrast, cells obtained from the tumor nodule or spleen of mice even 1 day after therapy with the high dose of L-PAM, 15 mg/kg, failed to establish tumors in any of the new recipients. Thus, the curative effect of the low dose of L-PAM, 0.75 mg/kg, is not due solely to its tumoricidal effect, whereas the curative effect of the high dose of L-PAM, 15 mg/kg, appears to be due primarily to the drug's tumoricidal effect.

Effect of Anti-Thymocyte Serum on Eradication of a Large Tumor Mediated by a Low or High Dose of L-PAM

Experiments were performed to determine whether host antitumor immunity is necessary for L-PAM-induced regression of large tumors (Table 4). Mice bearing a 21-mm tumor were treated with either a low dose of L-PAM (2.5 mg/kg) or a high dose of L-PAM (15 mg/kg), and then given three injections of rabbit anti-mouse thymocyte serum on days 2, 4, and 6 post therapy (Table 4). Treatment of tumor bearers with either a low dose or a high dose of L-PAM cured most of the mice. However, when low-dose L-PAM therapy was followed by treatment with anti-thymocyte serum, tumor regression was abrogated in most of the mice. On the other hand, anti-thymocyte serum given after a high dose of L-PAM did not reduce the effectiveness of the chemotherapy. Thus, in the MOPC-315 tumor system, tumor regression induced by a low but not by a high dose of L-PAM appears to require the participation of T-cell-dependent antitumor immunity.

Ability of Mice Cured by L-PAM to Reject a Lethal Challenge with MOPC-315 Tumor Cells

Initially, mice cured by a dose of L-PAM, 0.75, 2.5, or 7.5 mg/kg, given on day 11 after tumor inoculation were

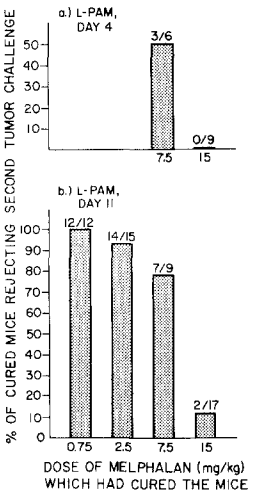

Fig. 6a and b. Ability of mice cured by various single doses of L-PAM to reject a lethal challenge. On day 60 post tumor inoculation with a 1×10^6 MOPC-315 tumor cells, mice cured of a nonpalpable tumor (day 4) (**a**) or of a large tumor (day 11, 20 mm) (**b**) were challenged with 1×10^6 tumor cells

challenged on day 30 after the first inoculation with 10^4, 10^5, or 10^6 viable tumor cells. All the cured mice resisted tumor challenge with any of the three tumor doses used, whereas all the control mice died.

In subsequent experiments, cured mice were challenged with 10^6 viable tumor cells (300 × the minimal lethal dose) on day 60 after the initial tumor inoculation (Fig. 6). Most of the mice cured by L-PAM in a dose of 0.75, 2.5, or 7.5 mg/kg given on day 11 rejected a challenge with tumor cells given on day 60 (Fig. 6b). However, mice cured by a high dose of L-PAM, 15 mg/kg, given on day 11 and challenged on day 60 developed tumors and succumbed (Fig. 6b). Similarly, the mice cured by the high dose of L-PAM, 15 mg/kg, given on day 4 also developed tumors and succumbed to a challenge given on day 60 (Fig. 6a). Finally, of the six out of 20 mice that were cured by a lower dose of L-PAM, 7.5 mg/kg, on day 4, three mice rejected the challenge given on day 60 and three mice succumbed (Fig. 6a). Thus, mice cured by lower doses of L-PAM are better able to reject a subsequent tumor challenge.

Discussion

We show here that the timing of the administration of a low dose of L-PAM (0.75 mg/kg) to MOPC-315 tumor-bearing mice is critical for successful therapy. Accordingly, a single low dose of L-PAM did not cure any of the mice bearing a MOPC-315 tumor at an early stage of growth (day 4) when the tumor was nonpalpable, yet this same dose of L-PAM did cure most (90%) of the mice bearing tumors at a late stage of growth when the primary tumor was large (day 11, 20 mm) and metastases were extensive. The eradication of the large tumor mediated by a low dose of L-PAM was not due solely to the drug's tumoricidal effect, but due also to the contribution of antitumor immunity of the host, which was apparently T-cell-dependent.

The single low dose of L-PAM was much less effective (30% cure) for mice bearing a large tumor and metastases if the mice had been previously treated with the same low dose of L-PAM when the tumor was nonpalpable. Thus, a low dose of L-PAM given at an early stage of tumor growth compromised the ability of mice to respond effectively to the same low dose of drug given when the tumor became large. Nevertheless, a nonpalpable tumor can be eradicated by a low dose of L-PAM, if present concomitantly with a large tumor (20 mm, day 12) on the contralateral side. One possible explanation is that the low dose of L-PAM is curative for mice bearing a nonpalpable tumor in the presence of augmented antitumor immunity induced by L-PAM in mice bearing a large tumor. If this is indeed the case, as it is with a low dose of CY [18, 21], then it might be possible to cure mice bearing a nonpalpable tumor with a low dose of L-PAM in conjunction with adoptively transferred immune spleen cells. Results obtained in a preliminary experiment using adoptive immunotherapy after a low dose of L-PAM therapy support this possibility.

We also show here that cure of MOPC-315 tumor-bearing mice with a low dose of L-PAM, 0.75 mg/kg, may offer an important advantage compared to cure with a high dose of L-PAM, 15 mg/kg. Mice cured by the high dose of L-PAM were susceptible to a lethal tumor challenge, in contrast to mice cured by the low dose of L-PAM, which resisted subsequent lethal challenge with 300 times the minimal lethal tumor dose. Cure by the high dose of the drug is probably due solely to the tumoricidal effect of the drug, since no viable tumor cells were found in the primary tumor site or in the spleen even 1 day after the therapy and anti-thymocyte serum did not reduce the curative effect of chemotherapy.

The results reported here follow the same pattern as those reported previously with regard to CY therapy of mice bearing MOPC-315 tumors [6, 13, 14, 18, 21]. The curative effectiveness of a low dose of L-PAM or of CY depends on the timing of drug administration. Accordingly, a low dose of drug which is not curative for mice bearing a nonpalpable tumor is curative for most mice bearing a large size tumor but only if they had not been previously treated with a low dose of drug when they had a nonpalpable tumor. With both alkylating agents, the low dose of drug which is curative for most mice bearing a large tumor represents less than 10% of the maximal tolerable dose of drug. The curative effectiveness of the low dose of drug depends on the participation of antitumor immunity in tumor eradication, and mice cured by the low dose of drug are able to reject a tumor challenge with 300 times the minimal lethal tumor dose. Mice bearing a large tumor can be cured by a high dose of drug; however, these mice are not resistant to subsequent tumor challenge. Thus, the resistance of the cured mice to a subsequent tumor challenge depends on the dose of drug used for chemotherapy.

The curative effect of a low dose of L-PAM for mice bearing a large MOPC-315 tumor is not due solely to the tumoricidal effect of the drug, since even 3 days post chemotherapy, i.e., long after clearance of the drug from the circulation, viable tumor cells were present in the primary tumor nodule as well as in the spleen. That antitumor immunity, which appears to be T-cell-dependent, aids in the eradication of the remaining tumor cells is evident from experiments in which the curative effect of L-PAM was abolished when the mice were treated with ATS. Mechanisms other than T-cell-dependent antitumor immunity may explain the finding that 20% of the mice were cured by low-dose L-PAM despite treatment with ATS. One such mechanism may be monocyte-mediated killing of tumor cells, which was reported to be enhanced following exposure to L-PAM [15].

The ineffectiveness of a low dose of CY for therapy of mice bearing a nonpalpable tumor, in contrast to its effectiveness for

mice bearing a large tumor, had been attributed to insufficient antitumor immunity at the early stage of tumor growth [18, 21]. However, the possibility still exists that the ineffectiveness of CY therapy for the nonpalpable tumor might also be due to a less efficient conversion of the functionally inactive CY into its active metabolites by hepatic mixed-function oxidases at an early stage rather than a late stage of tumor growth. This possibility need not be considered for L-PAM therapy, since L-PAM is the active form of an alkylating agent, i.e., it does not require enzymatic activation to exert its toxic activity [25, 29]. Thus it appears that the most important factor in curative chemotherapy with a low dose of drug is the contribution of a sufficient level of antitumor immunity for tumor eradication.

A low dose of L-PAM, 0.75 mg/kg, cured none of the mice treated on day 4 (nonpalpable tumor), 20% of the mice treated on day 7 (6 mm tumor), 90% of the mice treated on day 11 (20 mm tumor), and only 10% of the mice treated on day 14 (29 mm tumor). Thus, the effectiveness of therapy initially increased with time, to a maximum at day 11, and then decreased as the tumor became very large. A similar pattern of effectiveness of low-dose chemotherapy with progression of T1699 tumor growth has been observed by Radov et al. [26, 27]. In their experiments, L-PAM was not effective on day 7 (1.2 mm tumor), most effective on day 10 (6.4 mm tumor), and less effective on day 16 (18.6 mm tumor) [26]. Also, the effectiveness of low-dose chemotherapy has been attributed in both tumor systems to cooperation between toxic action of the drug and antitumor immunity. However, the immune status of the mice responding most effectively to low-dose chemotherapy differed in the two tumor models. In the T1699 tumor model, the mice exhibited concomitant antitumor immunity at the time of chemotherapy and, among the day 10 tumor bearers, chemotherapy was effective only for mice in which the chemotherapy did not cause a rapid suppression of the titer of existing antitumor antibodies or a rapid decrease in delayed hypersensitivity to T1699 [27]. On the other hand, in the MOPC-315 tumor system, the chemotherapy was most effective for tumor-bearing mice that did not exhibit concomitant antitumor immunity, as was evident from their inability to reject a tumor challenge (Table 2) and the inability of their spleen cells to lyse target cells in vitro in the 4-h ^{51}Cr-release assay and in vivo in the Winn assay [19]. Moreover, a low dose of L-PAM was curative for most MOPC-315 tumor bearers at a stage when suppressor cells that interfere with the generation and expression of antitumor immunity are active [13, 20], and drug-induced elimination or dilution of immunosuppressive activity is required for the immune system to aid effectively in tumor eradication. Finally, in spite of the concomitant antitumor immunity in the T1699 system due to the high immunogenicity of the tumor cells, Radov et al. obtained only transient tumor regression, the tumors reappearing within 30 days post chemotherapy [26]. In contrast, although the MOPC-315 tumor is weakly immunogenic [24, 28], low-dose chemotherapy caused tumor regression in most mice with no reappearance of tumor over the 50 days of observation post chemotherapy. Such mice were also resistant to a subsequent lethal tumor challenge. Although there are these important differences between the T1699 and MOPC-315 tumor systems, the results of Radov et al. with a different tumor (the T1699 mammary adenocarcinoma), the results previously reported from our laboratory with a different drug (CY), and the results presented here with L-PAM and the MOPC-315 plasmacytoma are all consistent in showing the importance of timing of drug administration in tumor chemotherapy.

L-PAM and CY show similar patterns of effectiveness with progression of MOPC-315 tumor growth. However, although the effectiveness of both drugs is mediated via their tumoricidal activity, their mechanism of enhancement of antitumor immunity might be different. L-PAM can increase the immunogenicity of MOPC-315 tumor cells [3] probably by acting as a hapten, thereby enhancing the recognition of tumor-associated antigen [2]. The fact that L-PAM, unlike CY, is active in its native form will allow us to study the direct effect of the drug not only on the tumor but also on the components of the immune system. Yamamura et al. analyzed in vitro the collaborative cytotoxicity between immune effector cells and melphalan and concluded that exposure of tumor cells to killer cells increases the susceptibility of the tumor cells to the tumoricidal effect of the drug [31]. However, at present, the mechanism of action of L-PAM as an immunomodulator remains obscure and requires further study [3].

Most mice treated on day 4 with a noncurative low dose of L-PAM, 0.75 mg/kg, were also not cured by a second, otherwise curative low dose of L-PAM given when the tumor became large. Some possible explanations of this phenomenon are as follows. The drug given at an early stage of tumor growth might cause a decrease in host antitumor immunity and/or induce a change in the biology of the tumor. Host antitumor immunity may decrease if the activity of helper T cells or cytotoxic cells is reduced or if the activity of suppressor cells is enhanced by the drug. The drug may also modify the tumor so as to decrease its immunogenicity or increase its resistance to immune lysis or the drug's tumoricidal effect. Since similar results were obtained previously with CY in treating mice with the MOPC-315 tumor and since in those experiments it was shown that the initial dose of CY at an early stage of tumor growth decreased the host's potential for the development of antitumor immunity [6, 18, 21], we suggest that this also occurs with L-PAM therapy.

Recently, Adler and Altbaum [1] evaluated the effectiveness of L-PAM therapy for mice bearing 5- to 15-mm MOPC-315 tumors. The L-PAM therapy consisted of multiple IT and/or IP injections of a relatively high dose of the drug (10–25 mg/kg), starting on the first day after inoculation of a relatively low dose of MOPC-315 tumor cells (1×10^4). Employing this intensive L-PAM therapy, they were able to cure only 43%–64% of the mice, and of these only 36%–57% (15%–36% of the L-PAM-treated mice) were resistant to a subsequent challenge with the minimal lethal tumor dose, 1×10^4 tumor cells. In light of our results, the low rate of cure can be explained by the administration of L-PAM at the 'wrong' time (day 1) and the relatively low resistance to subsequent challenge may be due to the employment of relatively high doses of L-PAM.

A common practice in clinical oncology is to use a relatively high dose of drug since a higher dose is more tumoricidal. However, in addition to often severe complications, high doses of drug are also more immunosuppressive and might not only decrease the antitumor immune potential of the host, which could have otherwise aided in the eradication of the tumor, but also increase the host's susceptibility to infection. Here we have shown that a very low dose of drug, i.e., less than 10% of the maximal tolerable dose, is curative for a mouse bearing a relatively large tumor and extensive metastases. In this situation, the tumor is eradicated not only by the direct tumoricidal effect of the drug but also by the contribution of potent antitumor immunity that develops as a result of the immunomodulatory effect of the drug. Further-

more, mice cured by the low dose of drug, in contrast to those cured by the high dose of drug, have long-lasting potent antitumor immunity which may be important for preventing the development of dormant tumor foci. Thus, our results indicate that it is important to consider not only the tumoricidal effect of a drug but also the immunomodulatory effect of a drug in developing an optimal protocol for chemotherapy.

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