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Deficiency in immunocompetence of mice cured from large MOPC-315 plasmacytomas by melphalan therapy*

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Summary. Mice cured from large MOPC-315 tumors by a single dose of melphalan, 7.5 mg/kg, were examined for up to 60 days after the drug treatment (71 days after the tumor inoculation) for their ability to respond to mitogenic stimulation, specific and nonspecific antigenic stimulation and for their susceptibility to inoculation with an unrelated tumor, L10 lymphoma. The response of spleen cells from cured mice to mitogenic stimulation by phytohemagglutinin or concanavalin A was slightly depressed at an early stage after the drug treatment. The allogeneic response against C57BL spleen cells and the antibody response against sheep red blood cells (SRBC) of spleen cells from cured mice remained below normal levels during the whole observation period. The deficiency in response to antigenic stimulation was found to be due to impairment in T-cell function. Cured mice were also deficient in their response to SRBC immunization (antibody and delayed-type hypersensitivity responses) and were more susceptible to inoculation with an unrelated tumor, L10 lymphoma, than normal, noninoculated mice. On the other hand, spleen cells of cured mice developed a highly specific cytotoxic response against target MOPC-315 tumor cells and the cured mice were resistant to challenge with an otherwise highly tumorigenic dose of MOPC-315. Thus, cured mice remained deficient for a long period of time in their response to MOPC-315-unrelated antigens but, at the same time, they showed a potent specific antitumor immunity potential in vivo and in vitro.

Introduction

We have reported that low-dose therapy with melphalan (L-phenylalanine mustard) cured mice bearing large MOPC-315 plasmacytomas [1, 3–5]. The cure was assumed to be due to cooperation between the tumoricidal effect of the drug and the emergence of a protective host-specific antitumor immune response [1, 4, 5, 9].

Mice bearing plasmacytomas were shown to be deficient in their ability to produce an immune response. Thus the antibody response potential was impaired [7, 11, 27], the response to mitogenic stimulation was decreased [6] and, in some cases, the immunoglobulin levels were reduced [12]. We found recently (not shown here) that in addition to B-cell functions, T-cell ability to respond to antigenic or mitogenic stimulation was also reduced. It was also found [4, 5, 18, 19, 26] that spleen cells from mice bearing MOPC-315 tumors are depressed in their ability to generate a specific cytotoxic response in vitro against target MOPC-315 tumor cells.

Cure by low-dose melphalan of mice bearing large MOPC-315 tumors leads to the development of a potent specific antitumor immune response, as shown by in vivo and in vitro tests [1, 4, 5, 9]. Thus, the low cytotoxic response potential of tumor-bearing mice was turned to a high cytotoxic response ability by low-dose melphalan therapy [4, 5, 9] and the cured mice were highly resistant to challenge with a tumorigenic dose of MOPC-315.

The aim of the present work was to investigate whether recovery of antitumor immune response potential by melphalan therapy in mice bearing MOPC-315 tumors is also associated with recovery of a general ability for immunocompetence. For this purpose, various T- and B-cell functions of mice cured from MOPC-315 tumors by melphalan therapy, and their susceptibility to an unrelated tumor were examined.

Materials and methods

Mice. Male Balb/c and C57BL mice, 8–12 weeks old, were supplied by breeding colonies of the Tel-Aviv University, Tel-Aviv, and of the Hebrew University, Jerusalem, Israel.

Tumors. The weakly immunogenic MOPC-315 plasmacytoma is maintained in our laboratory by serial s.c. inoculation of 1×10^6 tumor cells into syngeneic Balb/c mice, a dose that is at least 500 times the minimal lethal tumor dose and kills the mice in approximately 18 days. The tumors were allowed to grow for 10–11 days (i.e., until the size of the s.c. tumor was approximately 15 mm) before melphalan therapy. For the preparation of tumor cell suspensions, the tumor was excised and minced with scissors in RPMI-1640 medium (Gibco, NY, USA). Single-cell suspensions were prepared by mechanical disruption of the tissue as described previously [17], and the viability, as determined by trypan blue dye (0.1%) exclusion, always exceeded 85%. In some experiments (as mentioned in Re-

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sults), a B-cell lymphoma line, L10, was employed. This tumor occurred spontaneously in Balb/c mice and is immunologically unrelated to the MOPC-315 tumor. The tumor was maintained in our laboratory by serial i.p. injections of 5×10^4 tumor cells in Balb/c mice. The L10 tumor was kindly supplied by Dr. R. Laskov, the Hebrew University, Jerusalem, Israel.

Chemotherapy. For all experiments, mice weighing 20–25 g bearing 11-day (15-mm) MOPC-315 tumors were used. A stock solution of melphalan, 20 mg/ml in acid/alcohol (2 M HCl/95% EtOH, 1/5) was prepared immediately before use and diluted in phosphate-buffered saline (PBS) at the appropriate concentration of 7.5 mg/kg. A single i.p. injection in 0.5 ml was given. The mice were monitored for tumor regression and survival. Complete regression of the local tumors was observed on the 10th day after melphalan therapy. At this time, spleens, livers and lungs of melphalan-treated mice, but not those of untreated tumor-bearing mice, were free of tumor cells (results not shown here).

Spleen cells preparations. Single spleen cell suspensions were prepared by mechanical disruption and the viability, as determined by trypan blue dye (0.1%), always exceeded 95%. The spleen cells were washed three times in plain medium (serum-free) before use. A splenic T-enriched fraction was obtained by passage through a nylon-wool column [13]. Depletion from most macrophages was achieved by passage through a glass-wool column [16]. The glass-wool-nonadherent fraction was a T+B-enriched population. The glass-wool-nonadherent fraction (mostly macrophages) was eluted from the column by washing with warm (37° C) EDTA/PBS [16]. The T-cell population was removed from the intact spleen-cell population or from the T+B-enriched fraction by treatment with monoclonal anti-Thy1.2 antibody (F7D5, Booth, England) and fresh guinea-pig serum complement [2].

In vitro immunization. The procedure for generating and measuring the antitumor cytotoxicity in the spleens of mice against target MOPC-315 tumor cells has been described previously [17]. Briefly, responder spleen cells (75×10^6) were co-cultured with 2.5×10^6 target mitomycin C treated (50 µg/ml for 30 min) MOPC-315 stimulator tumor cells (or L10 tumor cells for comparison), in 250-ml tissue-culture flasks (Falcon Plastics, Oxnard, Calif) in a final volume of 50 ml RPMI medium supplemented with 5% fetal calf serum, 1% non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml). Fresh medium was prepared on the day of culture initiation, and 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Miss), was added to give a final concentration of 50 µM, prior to use (complete medium). The cultures were incubated for 5 days, the time required for the in vitro generation of optimal levels of anti-(MOPC-315) cytotoxicity [17].

Antitumor cytotoxicity assay. Cell-mediated immune lysis was determined as described previously, utilizing the 3.5-h ^{51}Cr release assay [17]. The percentage of specific ^{51}Cr release was calculated by the following formula: specific ^{51}Cr release (%) $= (T-C)/(M-C) \times 100$, where T is the percentage lysis of test spleen cells, C is the percentage spontaneous release by the target cells alone (which ranged between 17% and 25%), and M is the percentage maximal ^{51}Cr release obtained by three cycles of freezing and thawing (which ranged between 82% and 91%). The level of antitumor cytotoxicity is presented as means ± SE of the percentage specific ^{51}Cr release of six parallel samples at an E/T (effector/target) ratio of 100/1.

Mitogenic stimulation. Assays of lymphocyte stimulation were performed by a micromethod using an automatic harvester [21, 22]. Spleen cells were incubated for 3 days in RPMI medium supplemented with 10% fetal calf serum in flat-bottom Linbro plates at 37° C in a humidified atmosphere containing 10% CO_2. Phytohemagglutinin (Wellcome, Beckenham, England; 0.1 µg/culture), concanavalin A (Calbiochem; San Diego, Calif, USA; 0.25 µg/culture) or lipopolysaccharide (*E. coli* 055:B5; Difco, USA; 100 µg/culture) were added to spleen cell cultures containing 2×10^5 cells at the beginning of the incubation time. The quantitites of mitogens used were found to be optimal in preliminary experiments. [^{3}H]Thymidine (Nuclear Research Center, Negev, Israel; 1 µCi/culture) was added for the last 6 h of incubation.

Mixed lymphocyte reaction. Allogeneic stimulation of Balb/c splenocytes by C57BL spleen cells was determined [21]. C57Bl spleen cells were treated with mitomycin C (Sigma, St. Louis, Miss, USA; 50 µg/1×10^7 cells) for 30 min at 37° C. Equal quantities of 4×10^5 Balb/c spleen cells and of mitomycin-C-treated C57BL spleen cells were mixed and cultured in 200 µl complete RPMI medium. The cultures were incubated for 4 days in Falcon flat-bottom plates at 37° C. [^{3}H]Thymidine (1 µCi/culture) was added for the last 6 h of incubation. Each combination mixture was prepared in 12 parallel samples and the results (cpm) represent means ± SE.

Primary antibody response in vitro. The generation of a primary antibody response in vitro against a sheep red blood cell (SRBC) antigen entity was determined by a Micro-Marbrook method as described [10]. The "in" phase contained 2×10^6 spleen cells and 2×10^6 SRBC in a total volume of 200 µl, separated by a dialysis membrane from the "out" phase containing 1 ml RPMI complete medium. The cultures were incubated for 4 days at 37° C. The antibody response was evaluated by counting the number of specific-antibody-forming cells: [no. plaque-forming cells (PFC) in cultures with SRBC]−[no. PFC in corresponding cultures without SRBC]. The number of PFC was determined by a liquid hemolytic-plaque assay method [8].

In vivo immunizations. The primary antibody response in vivo to SRBC was determined by enumerating specific splenic PFC 4 or 5 days after i.p. injection of 1×10^9 SRBC in Balb/c mice. The delayed-type hypersensitivity (DTH) response was determined 4 days after i.v. injection of 1×10^6 SRBC in Balb/c mice. The DTH determinations were based on an evaluation of specific footpad swelling (footpad thickness before or after injection) 24 h after injection of 1×10^8 SRBC into the footpad. The swelling in the footpad injected with SRBC was compared with that measured in the adjacent pad injected with PBS alone [15].

Susceptibility to L10 tumor. Normal Balb/c mice or mice cured from MOPC-315 tumor by melphalan therapy were

inoculated i.p. with various doses of L10 tumor cells. The mice were monitored for survival until 100 days after inoculation.

Statistical analysis. The double-tailed Mann-Whitney *U*-test was employed.

Results

The response of mice cured from a large MOPC-315 tumor by melphalan was compared with that of normal noninoculated, untreated mice in various tests of mitogenic and antigenic stimulations in vitro and in vivo.

Table 1. Mitogenic stimulation of spleen cells from mice bearing MOPC-315 tumors cured by melphalan

Expt.	Time after melphalan[a] (days)	Mitogen added[b]	$[^3H]$Thymidine incorporation[b] (cpm) ± SE PHA	ConA	LPS
I	Control	−	1214 ± 101	1278 ± 231	1443 ± 401
		+	11 789 ± 678	11 195 ± 517	15 533 ± 619
	10	−	423 ± 87	1422 ± 178	1599 ± 851
		+	7706 ± 823 (69)[c]	10 055 ± 701 (87)	16 532 ± 721 (105)
II	Control	−	1153 ± 432	1281 ± 211	2197 ± 186
		+	10 507 ± 803	19 993 ± 651	17 893 ± 702
	15	−	2297 ± 501	1390 ± 311	2789 ± 313
		+	14 145 ± 817 (127)	15 187 ± 492 (74)	10 197 ± 769 (110)
III	Control	−	1141 ± 531	3301 ± 701	4141 ± 534
		+	14 412 ± 898	23 460 ± 7680	32 012 ± 822
	35	−	2151 ± 567	3431 ± 513	5003 ± 624
		+	17 699 ± 931 (117)	29 032 ± 7018 (107)	37 781 ± 911 (117)
IV	Control	−	1562 ± 218	2978 ± 723	2376 ± 227
		+	14 333 ± 751	29 191 ± 1801	38 272 ± 4402
	60	−	3771 ± 417	2781 ± 801	2425 ± 229
		+	21 505 ± 1566 (139)	30 821 ± 4801 (107)	45 100 ± 7658 (118)
V	Normal	−	700 ± 30		
		+	10 800 ± 150		
	9 (normal)	−	960 ± 35		
	Melphalan	+	9430 ± 1250 (87)		

[a] Control: normal, inoculated, untreated mice, belonging to the same batch as the inoculated mice and kept under similar conditions. Tumor-bearing mice were inoculated on day 0 with 1×10^5 MOPC-315 tumor cells, s.c. and treated on day 11 with 7.5 mg/kg melphalan i.p. when the tumor size reached approx. 15 mm; spleen cells were collected at various times after melphalan therapy: significant differences were found between control and the experimental group in Expt. I as regards. PHA and ConA stimulation and in Expt. II as regards ConA stimulation: $P < 0.002$, < 0.01 and < 0.02 respectively

[b] PHA, 0.1 μg/2×10^5 phytohemagglutinin; ConA, 0.25 μg concanavalin A; or LPS, 100 μg lipopolysaccharide/2×10^5 cells in each culture; each mitogen was added at the beginning of a 72-h incubation period; $[^3H]$thymidine, 1 μCi/culture, was added for the last 6 h of incubation; values (cpm) ± SE represent means of 12 parallel samples

[c] Percentage of control (parentheses) was calculated on the basis of "net" ^3H values: mean ^3H (cpm) in cultures with mitogen minus mean ^3H (cpm) in cultures without mitogen. Each of the experiments was repeated three times with similar results

Table 2. Allogeneic response of spleen cells from mice bearing MOPC-315 tumors cured by melphalan against C57BL spleen cells[a]

Expt.	Time after melphalan therapy (days)	$[^3H]$Thymidine incorporation[b] (cpm) ± SE Allogeneic mixture	Syngeneic mixture	Percentage of control[c] (%)	P^d
I	Control	42 101 ± 2011	2018 ± 118		
	15	24 602 ± 1871	1242 ± 121	58	<0.05
II[e]	Control	26 301 ± 671	2340 ± 89		
	39	15 318 ± 314	5471 ± 151	41	<0.05
III[e]	Control	27 301 ± 701	2351 ± 91		
	60	10 858 ± 408	3795 ± 84	28	<0.002

[a] See Table 1 for details on origin of spleen cells, tumor inoculation and melphalan therapy

[b] Culture period, 96 h; $[^3H]$thymidine (1 μCi/culture) added for the last 6 h of incubation; allogeneic mixture: 4×10^5 Balb/c spleen cells and 4×10^5 mitomycin-C-treated C57BL spleen cells; syngeneic mixture: 4×10^5 Balb/c spleen cells and 4×10^5 mitomycin-C-treated Balb/c spleen cells; mean radioactivity (cpm) ± SE of 12 parallel cultures

[c] Calculated by comparison of net ^3H values: ^3H (cpm) in allogeneic mixtures minus ^3H (cpm) in corresponding syngeneic mixtures, between control group and melphalon-treated-group of the same experiment

[d] *P* calculated by comparison between individual radioactivities in melphalan-treated and control groups of the same experiment

[e] Each of the experiments was repeated twice with similar results

Table 3. Primary antibody response in vitro to SRBC of spleen cells from mice bearing MOPC-315 tumors cured by melphalan[a]

Expt.	Time after melphalan therapy (days)	Mean specific PFC/10^6 spleen cells ± SE[b]	P[c]
I[d]	Control	88 ± 17	
	10	8 ± 2	<0.008
II	Control	36 ± 3	
	15	7 ± 5	<0.002
III	Control	36 ± 5	
	35	9 ± 2	<0.008
IV	Control	88 ± 7	
	57	11 ± 1	<0.008

[a] See Table 1 for details on origin of spleen cells, tumor inoculation and melphalan therapy
[b] Four days incubation; specific PFC: number of 19S direct plaque-forming cells in cultures with SRBC minus number of PFC in cultures without SRBC; means ± SE of 9 parallel samples
[c] *P* calculated by comparison between numbers of specific PFC generated in spleen cell cultures from cured mice and spleen cell cultures from corresponding control mice
[d] Expt. 1 was repeated twice with similar results

Mitogenic response potential of spleen cells from cured mice

The response to phytohemagglutinin mitogenic stimulation of spleen cells from cured mice was partially decreased 10 days after melphalan therapy, and later returned to normal levels. A slight decrease in the ability to respond to concanavalin A stimulation was observed 10 and 15 days after melphalan therapy. No decrease in the extent of lipopolysaccharide stimulation was observed, when assayed on various days after melphalan therapy. The results are presented in Table 1.

Allogeneic response potential of spleen cells from cured mice

As shown in Table 2, the response of Balb/c spleen cells from tumor-bearing mice cured by melphalan, against target C57BL spleen cells, was significantly lower than that of spleen cells from control, noninoculated, untreated mice. The allogeneic response remained low over the whole period of observation (up to 60 days after melphalan therapy).

Antibody response potential of spleen cells from cured mice

The primary antibody response in vitro to SRBC of spleen cells from cured mice was very low during the whole period of observation, i.e. from 10 to 57 days after melphalan therapy (Table 3). In view of these results, experiments were performed in order to determine whether the low antibody response potential was due to deficiency in macrophage, T- or B-cell functions. The antibody response in vitro to SRBC was determined in spleen cell cultures containing T+B cells from control, noninoculated mice and macrophages from either control mice or mice cured from MOPC-315 tumors by melphalan (Table 4). No significant difference was found between the PFC response in cultures supplied with macrophages from cured mice or from control mice. The PFC response was higher in cultures of whole spleen cells than in reconstituted ones but was still significantly lower in cultures of cells from cured mice than with cells from control mice. As shown in Table 5, the antibody response of B cells from cured mice was within the same range as that of B cells from control mice in cultures reconstituted with normal macrophages and T cells. On the other hand, T cells from spleens of cured mice were deficient in their helper function of generating an antibody response in cultures reconstituted with normal macrophages and B cells (Table 6). Accordingly, the decrease in potential for antibody response of spleens from the cured mice seems to be related to a deficiency in helper T-cell function.

Concomitant comparison of the specific cytotoxic response and a general immune response of cured mice

Previously reported results [4, 5, 9] have shown that spleens of mice cured from MOPC-315 tumors by melphalan therapy exhibit a very high specific cytotoxic response

Table 4. Primary antibody response in vitro to SRBC in cultures reconstituted with splenic macrophages from mice bearing MOPC-315 tumors cured by melphalan

Macrophage cells in culture[a] (×10^{-4})	Normal T+B splenocytes in culture[b] (×10^{-4})	Mean specific PFC/10^6 macrophages[c]		P
		"Normal" added	"Cured" added	
4	20	91 ± 8	90 ± 36	NS[d]
8	20	70 ± 25	77 ± 26	NS[d]
16	20	129 ± 46	82 ± 8	NS[d]
Control (whole spleen cells)		748 ± 112[e]	207 ± 34[f]	<0.002[g]

[a] Glass-wool-adherent (mostly macrophages) were obtained from spleens of normal, nonioculated, untreated mice or from tumor-bearing cured mice on the 37th day after melphalan therapy (day 11 after tumor inoculation)
[b] Splenic glass-wool-nonadherent (T+B-enriched) population from normal mice
[c] Specific PFC: number of PFC in cultures with SRBC minus number of PFC in cultures without SRBC; means ± SE of 9 parallel samples
[d] *P*, comparison between number of PFC in cultures with added macrophages from cured mice versus PFC in cultures with added macrophages from normal mice; NS, not significant
[e] Spleen cells from normal mice; no macrophages added
[f] Spleen cells from cured mice; no macrophages added
[g] Comparison between number of PFC in whole spleen cell cultures from cured mice and PFC in whole spleen cell cultures of normal mice. Experiment repeated three times with similar results

Table 5. Primary antibody response in vitro to SRBC of B cells from mice bearing MOPC-315 tumors cured by melphalan, in cultures reconstituted with normal macrophages (Mφ) and T cells

Normal Mφ and T splenocytes in cultures[a]		No. of B spleen cells[b] added ($\times 10^{-5}$)	Specific PFC/10^6 cells[c] in cultures containing		*P*
Mφ ($\times 10^{-4}$)	T ($\times 10^{-5}$)		"Normal" B	"Cured" B	
8	8	12	91 ± 20	62 ± 6	NS[d]
8	5	15	62 ± 12	43 ± 6	NS[d]
8	3	17	52 ± 10	43 ± 6	NS[d]
Control (whole spleen cells)			157 ± 21[e]	91 ± 20[f]	< 0.05[g]

[a] Mφ (glass-wool-adherent) and T cells (nylon-wool-nonadherent) were obtained from normal noninoculated, untreated mice
[b] B cells (glass-wool-nonadherent T-cell-depleted population) were obtained from normal or cured mice. "Cured" mice: mice treated with melphalan on the 11th day after tumor cell inoculation and spleen cells collected 37 days after the melphalan administration
[c] Means ± SE of 9 parallel samples
[d] Comparison between no. of PFC in cultures with B spleen cells from cured mice versus PFC in cultures with splenic B cells from normal mice; NS, not significant
[e] Spleen cells from normal mice; no B cells added
[f] Spleen cells from cured mice; no B cells added
[g] Comparison between no. of PFC in whole spleen-cell cultures from cured mice and whole spleen-cell cultures from normal mice. Experiments repeated with similar results

Table 6. Primary antibody response in vitro to SRBC in cultures reconstituted with splenic T cells from mice bearing MOPC-315 tumors cured by melphalan

Normal Mφ + B splenocytes in cultures[a] ($\times 10^{-5}$)	No. of T spleen cells added[b] ($\times 10^{-5}$)	Specific PFC/10^6 cells[c] in cultures containing		*P*
		"Normal" T	"Cured" T	
17	3	43 ± 9	25 ± 9	< 0.04[d]
14	6	62 ± 23	25 ± 9	< 0.04[d]
8	12	107 ± 12	49 ± 15	< 0.008[d]
Control (whole spleen cells)		207 ± 41[e]	13 ± 2[f]	< 0.004[g]

[a] Mφ + B (unfractionated spleen cell population depleted of T cells) were obtained from normal noninoculated, untreated mice
[b] T: nylon-wool-nonadherent population; cured: spleens taken 37 days after melphalan therapy
[c] Means ± SE of 9 parallel samples
[d] Comparison between no. of PFC in cultures with splenic T cells from cured mice versus no. of PFC in cultures with splenic T cells from normal mice
[e] Spleen cells from normal mice; no T cells added
[f] Spleen cells from cured mice; no T cells added
[g] Comparison between no. of PFC in whole spleen cell cultures from cured mice and whole spleen cell cultures from normal mice. Experiments repeated three times with similar results

Table 7. Concomitant determination of specific and general immunological responsiveness in vitro of mice bearing MOPC-315 tumors cured by melphalan[a]

Origin of spleen cells	Antitumor response (% of specific ^{51}Cr release)[b]	Antibody response (specific SRBC PFC/10^6 cells) means ± SE	Allogeneic response: [^{3}H]thymidine incorporation (cpm) ± SE		Percentage of control (%)
			Allogeneic mixture	Syngeneic mixture	
Normal	4.0 ± 2.0	164 ± 42	16803 ± 1173	2718 ± 455	
Tumor-bearing	36.2 ± 4.1	3.3 ± 0.7	10915 ± 1772	2119 ± 222	62.4
+ melphalan (4)[c]	0	13 ± 5.5	12507 ± 1058	1907 ± 282	75.2
+ melphalan (10)	58.2 ± 5.3	10 ± 2.0	13346 ± 638	1742 ± 101	82.3
+ melphalan (53)	90.3 ± 6.1	8 ± 1.8	12560 ± 274	2550 ± 226	71.0

[a] See Tables 1, 2 and 3 for details
[b] 5-day cultures; effector (spleen cells)/target (MOPC-315 cells) ratio: 100/1
[c] Numbers in parentheses show the time (days) after melphalan therapy. The same suspension of spleen cells was used for all tests in each group

Table 8. Primary antibody response to SRBC immunization of mice bearing MOPC-315 tumors cured by melphalan[a]

Expt.	Time after therapy[b] (days)	Specific PFC/10^6 spleen cells means ± SE[c]	P[d]
I	Control	162 ± 62	
	18	58 ± 7	<0.03
II	Control	192 ± 16	
	21	5 ± 2	<0.002
III	Control	260 ± 3	
	43	7 ± 2	<0.001

[a] Immunization by 1×10^9 SRBC i.p.; ten mice per group; spleen cells were collected 5 days after immunization (Expt. I) or 4 days after immunization (Expts. II and III)
[b] Control: noninoculated, untreated mice. Numbers show the time after melphalan therapy when tumor-bearing mice, treated with melphalan on the 11th day after tumor inoculation, were immunized with SRBC
[c] Means ± SE of 12 parallel samples; spleens from ten mice per group were pooled
[d] *P* calculated by comparison between specific PFC values in tumor-bearing melphalan-treated group and corresponding control group
Exp. no. III was performed 3 times with similar results

potential. In view of the present results showing a decrease in their ability to respond to various antigenic stimuli, an experiment was carried out in which spleen cells of cured mice originating from the same suspension were concomitantly tested for their specific, SRBC antibody and their allogeneic responses. As shown in Table 7, the antibody response to SRBC and the allogeneic response to C57BL spleen cells were found again to be depressed, whereas the specific cytotoxic response against MOPC-315 tumor cells of mice cured by melphalan therapy was markedly enhanced.

In vivo immune response of cured mice

As reported in the present work, the ability of spleen cells from MOPC-315 tumor-bearing mice, cured by melphalan, to respond to in vitro stimulation by SRBC or C57BL spleen cells was markedly depressed. Accordingly, it was of interest to determine whether this deficiency is also expressed in vivo as tested by the immune response to SRBC. The antibody response to SRBC of cured mice was markedly depressed (Table 8) and no delayed-type hypersensitivity response to SRBC could be elicited in the cured mice (Table 9).

Susceptibility of cured mice to L10 lymphoma

It was of interest to determine whether the deficiency in the overall ability of mice cured from MOPC-315 tumors by melphalan to respond to antigenic stimuli is also expressed by an increase in their susceptibility to inoculation with an unrelated tumor. For this purpose, experiments were done with the L10 lymphoma tumor. This tumor was shown not to be related immunologically to the MOPC-315 tumor, as indicated by a lack of cytotoxic response in vitro to this tumor by spleen cells possessing high cytotoxic response potential against the MOPC-315 target cells (Table 10). Moreover, mice cured from MOPC-315

Table 9. Delayed-type hypersensitivity (DTH) response to SRBC of mice bearing MOPC-315 tumors cured by melphalan[a]

Group	Foot pad	Mean fodpad thickness (mm)[b] ± SE		DTH response[c] (mm)
		Before injection	After injection	
Control	L	1.52 ± 0.03	1.49 ± 0.01	
	R	1.52 ± 0.03	1.67 ± 0.05	0.18
Tumor-bearing	L	1.50 ± 0.02	1.52 ± 0.02	
treated	R	1.50 ± 0.02	1.52 ± 0.02	0

[a] Groups of ten mice were sensitized with 1×10^6 SRBC i.v. Control: noninoculated, untreated mice. Tumor-bearing mice: treated with melphalan on the 11th day after tumor inoculation and sensitized with SRBC on the 41st day after melphalan therapy
[b] 1×10^8 SRBC in 0.05 ml was injected into the right (R) footpad of each mouse on the 4th day after the sensitization; 0.05 ml PBS was injected into the leftpad (L) on the same day
[c] Specific swelling (DTH response): difference between the R and L footpad at 24 h after the injection. The experiment was repeated three times with similar results

Table 10. Cytotoxic response of spleen cells from mice bearing MOPC-315 tumors cured by melphalan to target MOPC-315 and L10 lymphoma cells

Origin of effector spleen cells[a]	Tumor target cells	Antitumor cytotoxicity[b] (% specific ^{51}Cr release) means ± SE at E/T 100/1
Control	MOPC-315	1.8 ± 0.7
	L10	−1.6 ± 1.1
Tumor-bearing,	MOPC-315	61.1 ± 5.1
treated	L10	0

[a] Control: noninoculated, untreated mice. MOPC-315 tumor-bearing mice were treated with melphalan on the 11th day after inoculation and spleens were removed on the 70th day after melphalan therapy
[b] 5-day cultures; E/T: effector/target cell ratio 100/1 (5×10^6/5×10^4 cells); means ± SE of six parallel samples. The experiment was repeated twice with similar results

by melphalan were highly resistant to MOPC-315 challenge but were susceptible to inoculation with L10 lymphoma cells (Fig. 1). As shown in Fig. 2, mice cured from MOPC-315 tumor by melphalan were more susceptible to inoculation with various doses of L10 tumor cells than control, noninoculated mice.

Discussion

The main finding arising from the present work is that mice bearing MOPC-315 tumors, cured by melphalan therapy, are deficient in their ability to respond to antigenic stimulation in vitro and in vivo. On the other hand, they are highly resistant to challenge with MOPC-315 tumor cells and their spleens exhibit a high potential for generating a specific cytotoxic response against target MOPC-315 tumor cells.

A decrease in immunocompetence in mice bearing plasmacytomas has been described by various authors [6, 7, 11, 12, 27]. It has been also reported that spleen cells

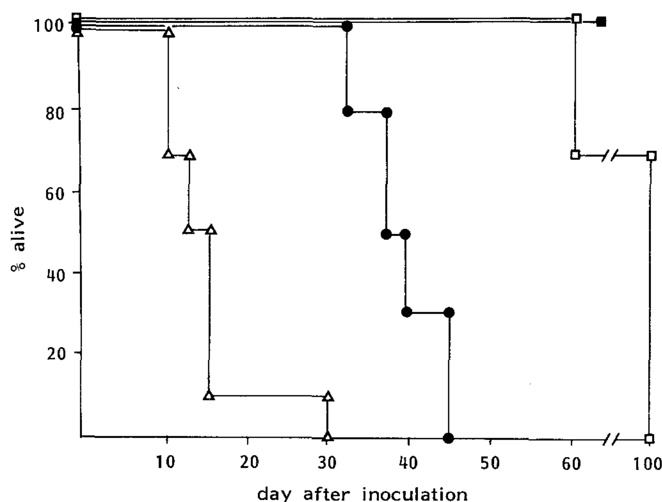

Fig. 1. Susceptibility to inoculation with MOPC-315 or L10 tumor cells of mice cured from MOPC-315 tumors by melphalan therapy. ■——■, Cured mice challenged with MOPC-315 tumor cells; ●——●, cured mice inoculated with L10 tumor cells; △——△, normal mice inoculated with MOPC-315 tumor cells; □——□, normal mice inoculated with L10 tumor cells. Ten mice per group; 7.5 mg/kg melphalan was injected on the 11th day after tumor inoculation; challenge with MOPC-315 or inoculation with L10 was done 40 days after melphalan therapy; normal mice, not previously inoculated with MOPC-315 and not treated with melphalan, belonged to the same batch as the melphalan-cured mice; MOPC-315: 2×10^6 cells s.c.; L10: 5×10^3 cells s.c.

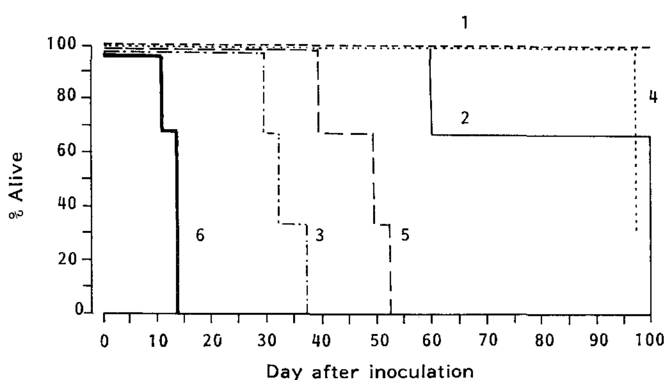

Fig. 2. Effect of inoculation with different doses of L10 tumor cells on either normal mice or mice cured from MOPC-315 tumors by melphalan therapy. 1, Normal mice inoculated with 5×10^2 L10 tumor cells; 2, normal mice inoculated with 5×10^3 L10 tumor cells; 3, normal mice inoculated with 5×10^4 L10 tumor cells; 4, cured mice inoculated with 5×10^2 L10 tumor cells; 5, cured mice inoculated with 5×10^3 L10 tumor cells; 6, cured mice inoculated with 5×10^4 L10 tumor cells

from mice bearing MOPC-315 tumors secrete a soluble factor that suppresses the induction of a primary antibody response in vivo to SRBC [14]. We found recently in our laboratory (results not shown here) that, at various stages of MOPC-315 tumor progression the mitogenic response as well as the response to antigenic stimulation is depressed. This applies to mitogenic stimulation by phytohemagglutinin and lipopolysaccharide at a late stage of tumor growth, to the allogeneic response against C57BL spleen cells, to the generation of a primary antibody response in vitro against SRBC and to the response in vivo to immunization with SRBC (antibody and delayed-type hypersensitivity responses). Moreover, it was reported that spleen cells from mice bearing MOPC-315 tumors are suppressed in their ability to generate a specific cytotoxic response in vitro against target MOPC-315 tumor cells [4, 5, 9, 18, 19]. The deficient response to mitogenic and antigenic stimulation of spleen cells from mice bearing MOPC-315 tumors was attributed to a decrease in T-cell function (mitogenic stimulation by phytohemagglutinin, antibody and allogeneic responses), emergence of suppressor T cells (allogeneic response) and impairment of B-cell function (lipopolysaccharide mitogeneic stimulation and antibody response).

The decrease in ability of spleen cells from mice bearing MOPC-315 tumors to respond to in vitro immunization with MOPC-315 tumor cells was attributed, at least partially, to the appearance of suppressive activity from splenic macrophages [4, 19, 26]. We have also shown that the natural-killer-type activity of spleen cells from mice with MOPC-315 tumors is markedly suppressed and that this suppression is mediated by macrophages [20]. In view of the low immunocompetence of mice bearing MOPC-315 tumors, it was of interest to determine the impact of cure by low-dose melphalan therapy on the immunocompetence status. It has been shown previously [1, 4, 5, 9], and we confirmed again, that cure by melphalan of mice bearing large MOPC-315 tumors results in development of a very potent specific antitumor immune response in vitro and in vivo. The effect of melphalan therapy in facilitating development of a potent antitumor immune response was attributed to its effect in abolishing the suppressive activity of splenic macrophages [4], in increasing the immunogenicity of tumor cells [5] and in promoting emergence of an immunopotentiating T-cell population in spleens and thymuses of tumor-bearing mice treated by the drug [25]. In regard to splenic NK-type activity, we have shown [20], that treatment with melphalan of mice bearing MOPC-315 tumors results in a short transient recovery, followed later by a return to a state of low NK-type activity in cured, tumor-free mice. The results presented in this paper on the immunocompetence status of mice cured from MOPC-315 tumors by melphalan should be evaluated in the light of known data on the immunocompetence of untreated, tumor-bearing mice. A striking difference between untreated tumor-bearing mice and mice cured by melphalan therapy lies in the selective effect of drug administration on the specific immune response (against MOPC-315 tumor cells) and on cell-mediated or antibody responses to unrelated antigens (allogeneic C57BL spleen cells and SRBC). Both specific and unrelated immune responses were markedly suppressed in untreated tumor-bearing mice, whereas in cured mice the specific antitumor immune response was markedly increased while the response to tumor-unrelated antigens was markedly suppressed. The low general immunocompetence of cured mice seems to be due to a deficiency in T-cell function, whereas previously reported results have shown suppressive activity of macrophages and impairment of both T- and B-cell functions in untreated mice bearing MOPC-315 tumors [4, 19, 26] (and our results not shown here).

It has been reported recently [23] that cure of mice bearing MOPC-315 tumors by oral administration of melphalan, resulted in prolonged impairment of mixed lym-

phocyte response activity, decrease in T-cell number and in interleukin-2 production. The T-cell deficiency of the cured mice was related to the previous presence of the tumor because noninoculated mice, submitted to the same schedule of melphalan administration, showed only transient reduction in T-cell activity. However, it should be mentioned that melphalan therapy had a dissociating effect on various immune responses. Thus, the antitumor immune response was augmented after melphalan administration in mice with progressively growing MOPC-315 tumors, whereas the antiviral response (against the C-type virus present in MOPC-315 tumor cells) was present in untreated tumor-bearing mice and abolished following tumor regression induced by melphalan therapy [24]. The assumption of prolonged overall T-cell deficiency because of the previous presence of the tumor does not explain the occurrence of a high, potent T-cell-mediated specific antitumor immune response in cured mice, as shown by previously reported results and by the results presented here, nor does it fit with the previously reported results [24] indicating an occurrence of anti-(C-type virus) response in untreated tumor-bearing mice. It seems that a deviation in the T-cell function towards a specific antitumor immune response at the expense of the T-cell response to unrelated antigens should also be considered.

Of special interest are the results obtained on the immunological responsiveness of cured mice to antigenic stimulation. The deficiency in antibody and delayed-type hypersensitivity response to SRBC of cured mice shows that impairment of the response of their spleens in in vitro tests is also expressed in a "real" situation of in vivo antigenic stimulation.

Mice cured from MOPC-315 tumors by melphalan therapy were also more susceptible to inoculation with an unrelated tumor, namely L10 lymphoma. As mentioned [20], cured mice remain deficient in the degree of NK-type activity of their spleen cells. It might be that one possible explanation for their higher susceptibility to an unrelated tumor is a low NK activity. This possibility, as well as the possibility that T-cell deficiency is also involved in the increase in susceptibility to an unrelated tumor, has yet to be investigated.

In conclusion: mice cured from large MOPC-315 tumors showed a potent specific antitumor immune response, whereas their response in vitro and in vivo to antigenic stimulation was markedly depressed, presumably because of a deficiency in their T-cell function. Moreover, the cured mice were more susceptible to inoculation with the unrelated tumor, L10 lymphoma, than normal, noninoculated mice.

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