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Immunotherapy of B-16 Melanoma with Peptidoglycan Monomer*

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Abstract—*B-16 melanoma-bearing mice received intravenously or intratumorally one or multiple injections of peptidoglycan monomer (PGM) derived from* Brevibacterium divaricatum *cell wall. Multiple injections of this non-toxic, water-soluble, low-molecular-weight peptidoglycan reduced the growth rate of tumor nodule on the leg, but did not significantly prolong the survival of tumor-bearing mice. One milligram of PGM administered* 3 *or* 7 days *after tumor inoculation inhibited formation of pulmonary metastases, induced either by intravenous injection of malignant cells or seeded spontaneously from tumor nodules in the legs before amputation. The inhibition reached about* 50% *of control values in saline-treated mice. Addition of PGM to* in vitro *cultures of B-16 melanoma cells did not change their growth rate. The phagocytic activity in the lungs, but not in the spleen and liver, was significantly augmented* 3 *and* 7 days *after treatment with PGM. These data indicate that the antimetastatic potency of PGM is probably due to activation of local (pulmonary) macrophages, and not due to direct cytotoxic effects on B-16 melanoma cells or to activation of systemic antineoplastic defence.*

INTRODUCTION

IMMUNOTHERAPY of malignant tumors with whole bacteria, like BCG or *C.parvum*, although effective in many instances entails significant side-effects. Bacterial cell walls, or fragments thereof, induce impressive cure rates in animals bearing experimental tumors [1–3], but have to be administered in oil; this requirement obviously limits clinical use of such preparations. Therefore a smaller component(s) of whole bacteria that would be non-toxic, possibly soluble in water and still with good antitumor effect was looked for. In this respect peptidoglycans from the bacterial cell walls are of particular interest.

Here we report about antimalignant effectiveness of peptidoglycan monomer (PGM), a non-toxic, water-soluble, low-molecular-weight peptidoglycan. PGM is a disaccharide-pentapeptide with a well-defined structure: GlcNac-MurNac-L-Ala-D-iso-Gln-meso-diaminopimelic acid-D-Ala-D-Ala [4, 5]. Previous studies have shown its *in vivo* immunostimulating activity [6, 7]. That led us to test for a possible antimalignant effect of the treatment with this peptidoglycan, using mice with B-16 melanoma as the experimental model. In this model the local tumor growth rate and metastases formation could be easily followed. Since the antimalignant effect of bacterial immunostimulators is most often attributed to the activation of macrophages [8–10], the phagocytic activity (local in the lungs and systemic in the spleen and liver) was tested in animals treated with PGM and compared with animals receiving no PGM.

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*The authors dedicate this article to Prof. Edgar Lederer on the occasion of his 75th birthday.

MATERIALS AND METHODS

Mice

Highly inbred mice of C57BL/H strain, 4–5 months old, were used. They were kept in standard plastic cages, fed with standard mouse food pellets and given water *ad libitum*.

Peptidoglycan monomer (PGM)

This was obtained by lysozyme digestion of uncross-linked peptidoglycan chains isolated from culture fluids of penicillin-treated *Brevibacterium divaricatum* NRRL-2311 [4, 5]. Immediately before administration by intravenous or intratumoral route PGM was dissolved in saline to make a 0.5% solution.

B-16 melanoma

This tumor has been maintained in our department for several years by successive subcutaneous transplantations. Single-cell suspensions of the melanoma were prepared by mincing the tumor tissue with scissors, forcing the brei through a nylon sieve and finally flushing the suspension through a thin-gauge needle. After subcutaneous transplantation of 4×10^6 cells into the thigh tumors appear in all recipients, which—if untreated—die in 30–50 days. The increase of the average diameter (mean of 2 perpendicular measurements) was used as a measure of the tumor growth rate.

Lung metastases

(*a*) *Induced metastases.* Mice received intravenously 2×10^5 melanoma cells and were killed 21 days later. Their lungs were excised and fixed in Bouin's fluid to allow easy detection of malignant nodules on the lung surface.

(*b*) *Spontaneous metastases.* Mice received 4×10^6 melanoma cells subcutaneously and 12 days later, when the tumor nodule was 0.8–1.0 cm in diameter, the tumor-bearing leg was amputated. The animals were killed 28 days after melanoma inoculation and the pulmonary metastases were counted.

Phagocytosis assay

To assay the phagocytic activity in the spleen, liver and lungs, the uptake of intravenously injected ^{51}Cr-labeled sheep erythrocytes was determined. Red cells (2×10^8), labeled as described in [11], were injected into mice treated with PGM or saline 1, 3 or 7 days before. Two hours later the animals were killed and the radioactivity of the organs was determined in a gamma-scintillation counter.

In vitro *cultures*

The procedure for setting up the primary cell culture was the same as described in [12]. Melanoma cells were grown in RPMI 1640 medium (Gibco, New York) supplemented with 10% fetal calf serum. Experiments were performed with cultures between the 3rd and 5th passages. L929 cells were grown in MEM 0011 medium (Eurobio, Paris) supplemented with 10% fetal calf serum. Cells were incubated at 37°C in humidified atmosphere containing 3% CO_2.

In vitro cytotoxic activity of PGM was assessed by determining the ability of treated cells for clonal growth. Three thousand melanoma cells (plating efficiency about 4.5%) or 2.5×10^2 L929 cells (plating efficiency about 70%) were seeded in plastic dishes and allowed to attach overnight. On the following day the cells were exposed to PGM dissolved in growth medium in the final concentration of 1.7, 17 or 50 μg/ml. Half of the cultures were incubated for 1 hr with PGM, washed twice with PBS and re-fed with fresh medium. The other half of the cultures were left in continuous contact with PGM. Colonies formed after 12 days of incubation were counted and the cytotoxic activity calculated.

Statistics

Differences between the results in experimental and in control groups of mice were evaluated by the *t* test or by the non-parametric Mann–Whitney *U* test.

The level of significance was set at 5% or less ($P < 0.05$).

RESULTS

Effects of intravenous administration of 1 mg of PGM on the survival of B-16 melanoma-bearing mice and on the growth rate of the tumor nodule are shown in Fig. 1. Repeated intravenous injections of PGM prolonged the survival of mice for a few days only (statistically not significant), but the tumor growth rate was significantly retarded. Likewise, intratumoral injections of PGM marginally prolonged the survival time of melanoma-bearing animals, although the tumor growth rate was again evidently slowed down (Fig. 2).

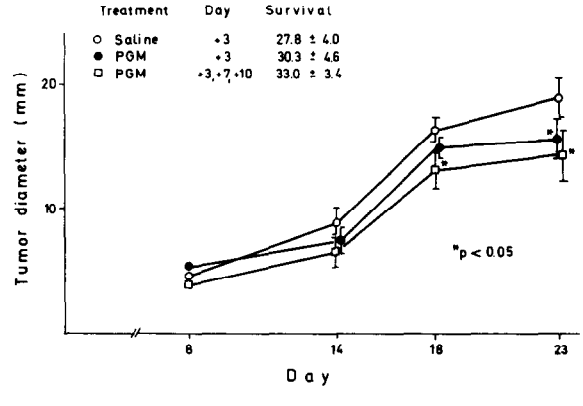

Fig. 1. Growth rate of subcutaneous B-16 melanoma nodules and survival time of tumor-bearing mice treated with PGM intravenously. There were 8–10 mice per group. Dose of PGM = 1.0 mg.

Effects of PGM treatment on the metastases formation were more impressive. Even one injection of 0.6 mg of PGM given 3 days after intravenous inoculation of melanoma cells reduced the number of induced pulmonary metastases to about 50% of the control value (Table 1). Multiple injections were not more effective than a single injection.

In animals with spontaneous metastases seeded

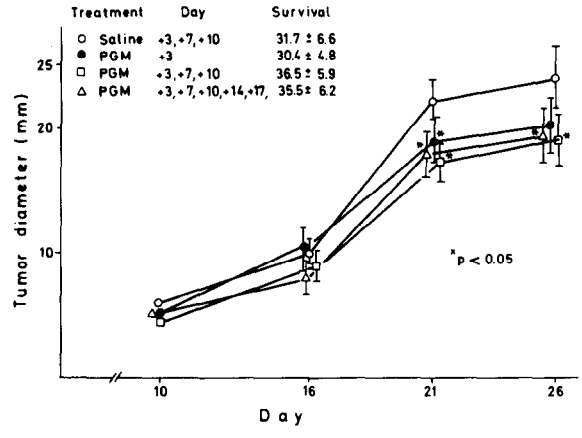

Fig. 2. Growth rate of subcutaneous B-16 melanoma nodules and survival time of tumor-bearing mice treated with PGM intratumorally. There were 8 mice per group. Dose of PGM = 1.0 mg.

from local tumor before its ablation, treatment with PGM 3 or 7 days after tumor implantation evidently reduced the number of pulmonary metastases (Table 2). Again, multiple injections of PGM were not superior to single-dose treatment. Treatment with PGM reduced the number of metastases only if administered early after tumor transplantation (3–7 days), while late administration (day 14) seemed to increase the number of metastases.

In order to test whether PGM causes direct toxic effects on melanoma cells and cultured fibroblasts, PGM was added in various concentrations to *in vitro* cultures of B-16 melanoma and of L929 cells. Even in maximal concentration (50 μg/ml), PGM did not reduce the growth potential of melanoma cells by more than 10% (Table 3). Lower concentrations of PGM in the culture medium, which could be compared with the doses effective *in vivo*, did not suppress the growth capacity of melanoma cells at all. L929 fibroblasts were even more resistant to PGM.

Finally, we assayed the phagocytic activity in the lungs, spleen and liver of mice injected intravenously with PGM 1, 3 or 7 days before the assay. As can be seen in Table 4, 1 day after PGM treatment there was no significant reduction, but 3 and 7 days after there was a significant increase in the phagocytic activity in the lungs. The phagocytosis in the spleen, as well as in the liver, remained practically unchanged after PGM injection.

DISCUSSION

The peptidoglycan monomer (PGM) used in this study belongs to a group of low-molecular-weight peptidoglycans, the most well-known representative of which is muramyl-dipeptide

Table 1. Number of induced pulmonary metastases of B-16 melanoma in mice treated with PGM intravenously

Expt	Treatment	Day	No. of mice	No. of metastases per mouse Range	x̄	%
1	Saline	3	10	16–21	22.6	100
	PGM, 0.3 mg	3	12	13–30	20.6	91
	PGM, 0.6 mg	3	8	5–19	11.7*	52
	PGM, 1.2 mg	3	10	6–18	12.0*	53
2	Saline	3, 7 and 10	10	9–29	19.7	100
	PGM, 0.3 mg	3, 7 and 10	12	12–22	15.3	78
	PGM, 0.6 mg	3, 7 and 10	12	6–13	9.2*	47
	PGM, 1.2 mg	3, 7 and 10	10	7.–18	12.4*	63

*$P < 0.05$.

Table 2. Number of spontaneous metastases of B-16 melanoma in mice treated with PGM intravenously

Expt	Treatment	Day	No. of mice	No. of metastases per mouse Range	x̄	%
1	Saline	3, 7 and 10	10	3–19	7.8	100
	PGM, 0.3 mg	3, 7 and 10	10	1–15	3.4*	44
	PGM, 0.6 mg	3	10	0–5	2.5*	32
	PGM, 1.2 mg	3	10	1–7	3.6*	46
2	Saline	7 and 14	12	4–9	4.8	100
	PGM, 0.3 mg	7 and 14	12	1–5	3.2	67
	PGM, 0.6 mg	7	12	1–3	2.0*	42
	PGM, 0.6 mg	14	12	1–13	7.3	152
	PGM, 1.2 mg	14	8	5–18	8.2	170

*$P < 0.05$.

Table 3. Clonal growth of B-16 melanoma and L929 cell cultures in the presence of PGM

Exposure to PGM	Dose of PGM (μg/ml)	Clonal growth (%) B-16 melanoma	L929
1 hour	0 (control)	100	100
	1.7	98	99
	17	97	95
	50	90	94
Continuous	0 (control)	100	100
	1.7	98	100
	17	96	100
	50	97	99

(MDP). MDP has been tested for antimalignant activity in various animal tumor models. Mouse macrophages treated with MDP *in vitro* acquired enhanced cytotoxic effectiveness against malignant cells [13, 14]. However, *in vivo* MDP proved to be active only if administered admixed to trehalose dimycolate [15, 16] or incorporated into liposomes [17]. Compared to this, our PGM administered without any additive showed: (a) a modest effect against local tumor (significant inhibition of the nodule growth rate, marginal prolongation of the survival of tumor-bearing animals); and (b) evident inhibition of induced and spontaneous pulmonary metastases. In a very similar experimental model (follow-up of the appearance of spontaneous pulmonary metastases in B-16 melanoma-bearing mice) treatment with MDP was ineffective [17]. This difference could be, perhaps, explained by the differences in biochemical structure of MDP and PGM. PGM is a larger molecule having one sugar (*N*-acetylglucosamine) and three amino acids (meso-diaminopimelic acid and two molecules of D-alanine) more than MDP.

Our *in vitro* studies suggest that PGM did not inactivate or kill B-16 melanoma cells directly. It was not toxic for L929 cells either. In addition, PGM added to *in vitro* cultures of lymphocytes did not influence the survival of these otherwise sensitive and fragile cells [18]. However, it should be kept in mind that like many solid tumors, B-16 melanoma comprises many different cell populations, especially regarding their capacity to form metastases [19]. *In vitro*, perhaps, only the most resistant cells acquire the ability to grow and such cells may be resistant to PGM as well. These cells might be quite different from the cells that form pulmonary metastases *in vivo*.

Nevertheless we prefer to correlate the observed antimetastatic effect of PGM treatment with activation of local, pulmonary macrophages. This view is supported by the finding of enhanced phagocytosis in the lungs of PGM-treated mice. Macrophages suppress formation of pulmonary

Table 4. Phagocytosis in the spleen, liver and lungs of mice 1, 3 and 7 days after intravenous treatment with 1.0 mg of PGM or saline

Day after treatment	Saline						PGM					
	Spleen		Liver		Lungs		Spleen		Liver		Lungs	
	A	B	A	B	A	B	A	B	A	B	A	B
1	660 ± 137	9.2 ± 2.9	4272 ± 418	4.64 ± 0.7	115 ± 25	0.97 ± 0.37	514 ± 98	7.5 ± 1.7	4049 ± 412	4.13 ± 0.42	80 ± 25	0.73 ± 0.23
3	673 ± 40	7.6 ± 0.5	3826 ± 237	4.07 ± 0.25	31 ±7.7	0.20 ± 0.07	524 ± 80	5.9 ± 0.9	4463 ± 364	4.52 ± 0.18	88 ± 19*	0.58 ± 0.12
7	506 ± 152	6.4 ± 1.6	4874 ± 833	4.94 ± 0.7	24.6 ± 14.3	0.12 0.09	675 ± 128	8.30 ± 2.5	4275 ± 776	4.04 ± 0.9	56 ± 13*	0.43 ± 0.10

Numbers represent total radioactivity per organ in counts/min (A) and radioactivity per mg of tissue (B) 2 hr after injection of ^{51}Cr-labeled sheep erythrocytes. There were 8 mice per group.
*$P < 0.05$ in comparison to control (saline).

metastases in general [20, 21] and formation of B-16 melanoma metastases in particular [22]. Absence of activation of splenic and liver macrophages at the same time when the pulmonary macrophages were stimulated was an unexpected finding. After intravenous injection PGM is very rapidly excreted from the organism, partly unchanged, and partly split into sugar and peptide moieties [23], but lungs, as the first capillary bed receiving intravenously inoculated material, are likely to receive a major portion of the original material. This might be one reason why splenic and hepatic macrophages were not stimulated with PGM. Another possibility would be that pulmonary macrophages are more susceptible to activation by bacterial peptidoglycans, but we have no data in support of this speculation.

Besides macrophages, natural killer (NK) cells might also be targets for PGM action. NK cells are involved in destruction of blood-borne B-16 melanoma metastases [24], and peptidoglycans like MDP activate NK cells in C57BL mice, the animals in which this tumor grows [25].

Our finding that only one injection of PGM was sufficient to suppress the appearance of metastases while additional injections did not considerably improve this effect might contradict the finding that *in vitro* activation of macrophages with MDP was significantly higher after repeated contact with this peptidoglycan [26]. We believe that our result simply indicates particular sensitivity of early steps in metastasis formation to the action of activated macrophages, while later steps are less sensitive. This time-dependence was shown in the treatment of Lewis lung carcinoma metastases by *C. parvum* [27].

Many data stress the importance of the route of administration of bacterial immunoadjuvants in the therapy of tumors. For effective treatment of pulmonary metastases, BCG cell walls have to be administered intravenously, while trehalose dimycolate (a glycolipid from bacterial cell walls) was most effective if administered intraperitoneally [28]. Antimetastatic potential of BCG cell walls could be correlated with granuloma formation in the lungs, and the activity of trehalose could be connected with systemic activation of the antitumor resistance. Our PGM inhibits metastasis formation only after intravenous administration and, according to the data presented in this paper, probably by activating local pulmonary resistance to metastasis formation.

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