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ENDOTOXIN-INDUCED ANTITUMOR ACTIVITY IN THE MOUSE IS HIGHLY POTENTIATED BY MURAMYL DIPEPTIDE

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SUMMARY

The ability of aqueous solutions of various endotoxin preparations, muramyl dipeptide (MDP) and combinations of endotoxin and MDP, to induce necrosis and regression of subcutaneous Meth A transplants in mice and their toxicity were studied. While intravenously injected toxic endotoxins, in contrast to a detoxified preparation and to MDP, induced considerable necrosis and regression of their own, addition of MDP potentiated the antitumor potential of both toxic and detoxified endotoxins to the same high degree. Detoxified endotoxin combined with MDP, however, was far less toxic than toxic preparations alone or combined with MDP. This indicates that toxicity is not directly related to therapeutic potential.

INTRODUCTION

Endotoxins are well known inducers of necrosis and regression of solid tumors in animals but they are too toxic for clinical application. Ribi et al. [10] recently showed that detoxified endotoxin preparations had potent antitumor activity against line 10 hepatocellular carcinoma in guinea pigs on intralesional administration, provided they were combined with trehalose dimycolate, a bacterial peptide fraction and oil. In the same model synthetic MDP was shown to potentiate the activity of a toxic endotoxin preparation [12]. As far as we know other therapeutic animal-tumor models have not been studied. We studied the effect of intravenously (i.v.) injected aqueous solutions of MDP in combination with various endotoxin preparations against subcutaneous (s.c.) Meth A fibrosarcoma transplants in syngeneic BALB/c mice.

MATERIALS AND METHODS

Mice and tumor

Female BALB/c mice, 11 weeks old, bred in our own facilities, were

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used. The Meth A sarcoma of BALB/c origin was obtained from the Clinical Research Centre (Harrow, Middlesex, U.K.) and maintained in the ascites form by serial intraperitoneal passage.

Materials

MDP was bought from the Institut Pasteur Production (Marnes-la-Coquette, France). Endotoxin from *E. coli* 0111:B4 (Difco Laboratories, Detroit, Michigan, U.S.A.) was prepared according to the phenol/water extraction method [16,17]. Refined toxic and detoxified endotoxin from the heptose-less (Re) mutant *Salmonella typhimurium* were obtained from Ribi ImmunoChem Inc. (Hamilton, MT, U.S.A.) and were prepared and refined as described by Ribi et al. [10]. In brief, endotoxic glycolipids were extracted from cell walls with phenol/chloroform/petroleum ether, fractionated by preparative microparticulate gel chromatography and further purified on Sephadex LH-20. This yielded disaggregated endotoxic glycolipids which were free of phospholipids and nucleic acid and had a protein content of less than 0.3%. The refined endotoxin was detoxified by hydrolysis in 0.1 N HCl at 100°C [10]. The detoxified preparation is virtually free of KDO and has about one-half of the phosphate content of the refined endotoxin. It has an LD_{50} of >10 mg/mouse [13].

MDP was dissolved in pyrogen free saline. Endotoxins were dissolved in 0.5% triethylamine (2 mg endotoxin/0.4 ml) and diluted in saline. Agents were injected i.v., alone or mixed, in a total volume of 0.5 ml.

Tumor assay

Mice were injected s.c. in the abdomen with 3×10^5 viable Meth A cells and treated 9 days later (tumor diameter ± 7.5 mm). Necrosis was measured at day 11 and expressed as 100 times the ratio of the mean diameters of necrotic area and tumor (extent). Incidence of dark and light stained necrosis were indicated separately. Growth inhibition was scored when tumor size did not increase within 2 days of injection. Complete cure was scored when tumors disappeared completely within 19 days of treatment.

RESULTS AND DISCUSSION

Injection of 25 μg *E. coli* endotoxin into BALB/c mice with 9-day-old Meth A tumors induced 100% incidence of extensive tumor necrosis followed by growth inhibition of most tumors and complete cure in 30% of the mice (Table 1). This activity pattern has been observed before [4,5]. Lower doses were less active in all respects. MDP alone in a range of doses did not cause tumor necrosis, but induced some regression. Addition of 3—100 μg of MDP to 10 μg *E. coli* endotoxin potentiated considerably all parameters of the antitumor activity of endotoxin measured. With exception of the extent of necrosis no clear dose-effect relation could be observed. Also, decreasing the amount of endotoxin while keeping the amount of MDP constant

TABLE 1

ANTITUMOR ACTIVITY OF *E. COLI* ENDOTOXIN AND/OR MDP[a]

Treatment		No. of mice	Necrosis			Incidence (%) of	
Endotoxin dose (μg)	MDP dose (μg)		Incidence (%)		Extent[b]	Growth inhibition	Complete cure
			Light	Dark			
25	0	10	30	70	57 ± 5	80	30
10	100	10	0	100	75 ± 4	100	90
10	30	20	0	100	68 ± 2	100	80
10	10	5	0	100	66 ± 3	100	100
10	3	5	0	100	57 ± 3	100	80
10	0	20	50	35	46 ± 3	65	15
3	30	10	0	100	61 ± 4	100	60
3	10	10	0	90	66 ± 2	100	60
3	0	14	50	7	49 ± 3	43	7
1	30	5	0	100	63 ± 5	100	80
1	0	5	40	0	36 ± 1	0	0
0.3	30	5	20	80	56 ± 7	100	60
0	100	10	0	0		30	20
0	30	15	0	0		7	7
0	10	4	0	0		25	25
0	3	5	0	0		0	0
0	0	20	0	0		5	0

[a] See Materials and Methods.
[b] Mean ± S.E.M.

(30 μg) did not result in a clear reduction of antitumor activity. In further experiments the effect of MDP on the antitumor activity of toxic and detoxified endotoxin of *S. typhimurium* Re was compared (Table 2). The detoxified *S. typhimurium* endotoxin caused very moderate necrosis in a range of doses, which confirms earlier data with low toxic KDO-poor lipid A from the same mutant and Sarcoma 37 in mice [15]. Also it hardly affected tumor growth. The toxic endotoxin showed considerable activity in a dose of 10 μg, but addition of MDP was frequently lethal. On the other hand the detoxified preparation acquired very potent activity in combination with MDP. It was questioned, however, whether the increase in therapeutic activity was accompanied by an increase of toxicity as earlier reports showed that MDP increased lethality of a toxic but not of a detoxified endotoxin preparation in the guinea pig [10,11]. This was partially confirmed in tumor-bearing mice by the lethality observed after combined administration of toxic endotoxin of *S. typhimurium* Re and MDP (Table 2). Therefore the effect of MDP on the toxicity of both *S. typhimurium* Re

TABLE 2

ANTITUMOR ACTIVITY OF TOXIC AND DETOXIFIED ENDOTOXIN OF *S. TYPHIMURIUM* Re[a] ALONE OR WITH MDP

Treatment		*n*	Necrosis			Incidence (%) of	
Dose (μg)	MDP dose (μg)		Incidence (%)		Extent	Growth inhibition	Complete cure
			Light	Dark			
S. typhimurium Re (detoxified)							
50	0	5	20	0	31	0	0
25	0	10	60	0	36 ± 6	10	0
10	30	10	10	90	61 ± 3	80	70
10	0	10	10	0	47	40	10
S. typhimurium Re							
10	30	5[b]	20	80	73 ± 4	100	80
10	0	10	50	50	57 ± 5	90	40
Saline							
0	30	10	0	0		0	0
0	0	10	0	0		0	0

[a] See Materials and Methods.
[b] Of 10 mice treated 5 died within 24 h.

preparations was investigated (Fig. 1). None of the combinations of detoxified endotoxin and MDP studied, caused lethality and diarrhea, while lethargy was only observed after higher doses of MDP. Weight loss increased with increasing doses of both agents but never exceeded 10%. Body weight by 48 h was always higher than by 24 h after treatment probably indicative of recovery. All doses of toxic endotoxin whether or not combined with MDP caused lethargy and diarrhea. Surprisingly MDP tended to protect against diarrhea. Weight loss was severe, sometimes over 15%, and higher by 48 h than by 24 h. The decreased weight loss observed after higher doses of MDP may reflect fluid accumulation in the tissues as a consequence of shock. Lethality was observed upon increasing the dose. While the LD_{50} of toxic endotoxin alone is over 160 μg per mouse, LD_{50} upon combination with 30 μg and 100 μg of MDP is 28.3 μg and 24.6 μg, respectively, as calculated by a modified Spearman-Karber method described by Nowotny [7]. The combined administration of 80 μg detoxified endotoxin and 30 μg MDP, however, was not lethal and even did not cause diarrhea and lethargy. The high lethality observed in tumor-bearing mice after injection of 10 μg of toxic endotoxin and 30 μg of MDP (Table 2) is probably due to the increased susceptibility of tumor-bearing mice to the toxic effects of endotoxin [1,8].

The results show that MDP enhances the toxicity of both endotoxin

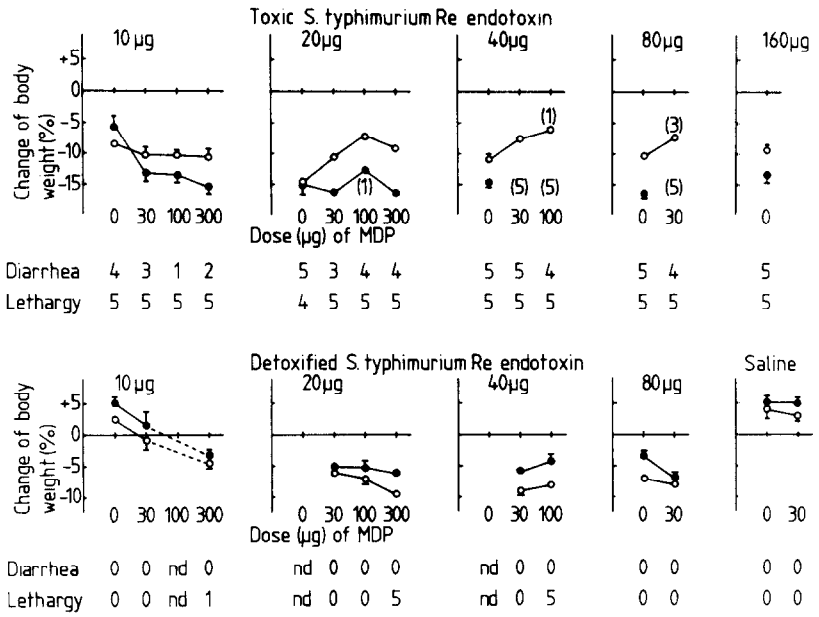

Fig. 1. Toxicity of i.v. injected toxic and detoxified *S. typhimurium* Re endotoxin administered alone or mixed with graded doses of MDP. Groups of 5 mice, provided with lab chow and water ad libitum, were kept at constant temperature (18°C) and humidity. Change of body weight 24 h (○) and 48 h (●) after injection was expressed proportionally to the individual weights immediately before injection. Bars indicate S.E.M. Figures between brackets indicate the total number of dead mice by 24 h and 48 h. Diarrhea and lethargy were scored by 5 h. Figures indicate the number of mice with these symptoms.

preparations, but that combined administration of MDP and detoxified endotoxin is far less toxic although it showed equal tumor-therapeutic activity. These data confirm and extend those of Ribi et al. [12] that a direct relation between toxicity and therapeutic effectiveness is absent. Because Ribi et al. [12] found that endotoxins from wild-type bacteria were not active in their system, they suggested that MDP would act as an adjuvant to antigen(s) exposed on endotoxin from rough-mutant bacteria but not on endotoxin from wild-type bacteria. These antigen(s) would cross-react with tumor antigens. The immunostimulation combined with the endotoxin-induced tumor necrosis would result in high cure rates. His explanation, however, is not supported by our results. A predominant adjuvant action of MDP for determinants only exposed on rough-type endotoxins is not likely in the mouse model, because both wild- and rough-type of endotoxin were active. Moreover, MDP potentiated the antitumor activity in a wide range of doses (Table 1), while the adjuvant activity of MDP is known to be rather dose-dependent [14]. Also, neither addition of oil, needed for cellular adjuvanticity of MDP [6], nor intralesional

administration were necessary to induce regression of Meth A sarcoma. As endotoxin-induced tumor regression was shown to be dependent on an intact T cell immune system and on the presence of concomitant immunity at the time of endotoxin injection [2,9], an immunopotentiating action of MDP remains likely. On the other hand the induction of tumor necrosis was shown to be an immune-independent event [2,9], possibly mediated by vasoamines and the adrenergic system [3,4]. The observed strong potentiation of the necrotizing activity of endotoxins by MDP, which itself lacked necrotizing activity, suggests that at least also a non-immunologic mechanism underlies the potentiation of the antitumor activity of endotoxins by MDP. Further studies are needed to explain the potentiating and/or synergistic activity of MDP and endotoxins.

The main conclusions that follow from our work are that detoxified endotoxin has only minor antitumor activity, but that this can be enhanced considerably on combination with MDP. As this is not accompanied by a strong increase of toxicity, clinical application of combination treatment may become feasible in the future.

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