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Immunotherapy of Experimental Cancer with a Mixture of Synthetic Muramyl Dipeptide and Trehalose Dimycolate

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The antitumor activity of a mixture of synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and trehalose-6,6'-dimycolate (TDM) (MDP+TDM) in emulsified form was studied in guinea pigs, each with a syngeneic dermal tumor and microscopically detectable metastases in regional lymph nodes. A single intralesional administration of an ultrasonically prepared emulsion containing MDP+TDM in squalane or in mineral oil caused tumor regression and elimination of lymph node metastases. Similar emulsions of MDP+TDM made with squalene or hexadecane were immunotherapeutically inactive.

The administration of living BCG, putatively a form of immunotherapy for cancer patients, sometimes has caused the unwanted complication of disseminated mycobacterial infection(s). Therefore, nonliving, immunotherapeutically active mycobacterial preparations have been tested and continue to be tested in experimental and clinical trials of cancer immunotherapy. The main objective of these studies is to identify mycobacterial components with maximal antitumor effects and minimal toxicity.

Trehalose-6,6'-dimycolate (TDM, known also as cord factor or P_3), a mycobacterial glycolipid (3), was found to be effective in regression of an established murine fibrosarcoma (9). In guinea pigs, emulsified TDM was immunotherapeutically ineffective unless it was admixed with endotoxin (8).

Synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine, known also as muramyl dipeptide (MDP), was found to be the minimal adjuvant active structure capable of replacing whole mycobacterial cells in complete Freund adjuvant for inducing hypersensitivity and increased antibody production to a variety of antigens (1, 2). This compound and several analogs have been synthesized and tested for antitumor activity. None of them has been found to be therapeutically effective against the guinea pig line 10 hepatoma (5, 7). Some of them in admixture with TDM have been found to be active. The compound containing the L-alanyl-D-isoglutamine residue was reported to be ineffective unless, in addition to TDM, an endotoxin component (B_4) was incorporated in the emulsion. The studies reported here demonstrate that this compound in admixture with TDM (without B_4) in emulsified form causes regression of dermal tumors and elimination of lymph node metastases in guinea pigs.

MATERIALS AND METHODS

Animals. Adult male or female guinea pigs, Sewall Wright strain 2, were obtained from stock maintained at the Frederick Cancer Research Center, Frederick, Md.

Compounds tested. TDM {mp, 40 to 42°C; $[\alpha]_D$ = +35°C ($CHCl_3$)} from *Mycobacterium bovis* AN5 (Institut Pasteur) was isolated by A. Escaut at the Pilot Plant of the Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. TDM was found to be free of endotoxin by the *Limulus* amoebocyte lysate test.

Synthetic MDP was obtained from G.I.R.P.I., Paris, France.

Preparation of compounds for emulsification. TDM in chloroform solution was added to dry MDP in a polypropylene tube (12 by 75 mm, Falcon Plastics, Oxnard, Calif.) if the emulsion was to be prepared by ultrasonication or in a glass tube of a tissue grinder equipped with a Teflon pestle if the emulsion was to be prepared by grinding. The solvent was evaporated, and the fractions were further dried at 37°C overnight. The appropriate amount of oil (squalane $C_{30}H_{62}$, squalene $C_{30}H_{50}$, hexadecane $C_{16}H_{34}$, or mineral oil [Drakeol 6VR]) was added, and the mixture was heated at 56°C for about 5 min to dissolve the TDM in oil.

Preparation of emulsions by ultrasonication. Ultrasonication (Ultrasonic Cleaner, model CC.-25; Beckman Instruments, Inc., Fullerton, Calif.) was applied for 1 or 2 min to disperse the mixture in oil. NaCl (0.15 M, 0.5 ml) containing 0.2% Tween 80 was added to the tube containing the mixture in oil, and ultrasonic treatment was reapplied for 2 min. The saline-Tween solution (0.4 ml) was added to the emulsion, and ultrasonic treatment was reapplied for 1 min. The emulsion was diluted with saline-Tween solution to the desired concentration of oil. The final concentrations of emulsion components were as follows: MDP, 0.75 to 0.0025 mg/ml; TDM, 2.5 to 0.0025 mg/ml; oil (squalane, squalene, hexadecane, or mineral oil), 10 or 2%; and Tween 80, ca. 0.2%. Emulsions containing MDP alone or TDM alone were prepared similarly.

Preparation of emulsions by grinding. The mix-

ture was ground for 30 s to disperse it in oil. A 0.15 M solution of NaCl containing 0.2% Tween 80 was added to the tube containing the mixture in oil, which was then ground for 2 min. The final concentrations of emulsion components were as follows: MDP, 0.75 mg/ml; TDM, 2.5 mg/ml; squalane, 10%; Tween 80, ca. 0.2%. Emulsion alone was prepared similarly.

Guinea pig tumor line and treatment. A line 10 tumor, an ascitic variant, was derived from a hepatocarcinoma induced by diethylnitrosamine in a strain 2 guinea pig. Inoculation of 10^6 tumor cells intradermally resulted in progressive local tumor growth, development of microscopic metastases in the draining lymph node by 1 week, and death 2 to 3 months after tumor cell inoculation (6). Animals were treated by intralesion injection of emulsified compounds in 0.4-ml volumes 7 days after tumor implantation, when the average diameter of the dermal tumors was about 10 mm. Tumor incidence was determined by weekly observations for 2 months. Treated animals whose dermal tumors regressed completely and that had no grossly detectable metastatic disease were rechallenged with 10^6 line 10 tumor cells inoculated intradermally on the contralateral flank. Injection sites were observed for 30 days thereafter for growth of rechallenge inoculum.

Statistical evaluation. Differences among groups were evaluated statistically by a 2 by 2 contingency table of the Fisher exact test.

RESULTS

In a preliminary experiment, we tested the ability of emulsions containing MDP+TDM to bring about regression of the dermal tumor and its lymphatic metastases. It was found (Table 1, experiment 1) that intralesional injection of emulsions containing a mixture of MDP and TDM (MDP+TDM) at sufficiently high concentrations brought about a cure rate of 100% whether the emulsions were prepared by ultrasonication or by grinding. The tumor grew progressively in the control animals treated intralesionally with emulsion lacking MDP+TDM.

In the next experiment, we compared the efficacy of an emulsion containing MDP+TDM in 10% squalane with one containing MDP+TDM in 2% squalane. We observed that both emulsions were highly effective in causing complete regression of the tumor (Table 1, experiment 2). Emulsions of MDP alone or TDM alone as well as emulsions lacking these compounds were not effective. Another experiment (Table 1, experiment 3) demonstrated that when an emulsion containing MDP alone was mixed with an emulsion containing TDM alone, the resulting mixture did not cause tumor regression after intrale-

TABLE 1. *Tumor regression induced in guinea pigs by intralesional injection of MDP+TDM emulsified in squalane*

Expt	Group	Material injected		Squalane concn (%)	Emulsification method[a]	No. of tumor-free[b] animals/ no. tested (at 90 days)	P[c]
		MDP (mg)	TDM (mg)				
1	1	0.3	1	10	G	7/7	<0.001
	2	0.3	1	10	US	7/7	<0.001
	3			10	US	0/8	
2	1	0.3	1	10	US	8/8	<0.001
	2	0.3	1	2	US	9/10	<0.001
	3	0.3		10	US	0/10	
	4		1	10	US	1/10	NS
	5			10	US	0/10	
3	1	0.3	0.1	2	US	6/6	<0.001
	2[d]	0.3		2	US	0/6	
			0.1				
	3			2	US	0/6	
4	1[e]	0.1	0.2	2	US	8/8	<0.001
	2	0.1	0.2	2	US	8/8	<0.001
	3[d]	0.1		2	US	0/8	
			0.2				

[a] G, Grinding; US, ultrasonication.

[b] Complete disappearance of the dermal tumor, no clinical evidence of metastatic disease, and rejection of contralateral challenge (10^6 tumor cells) 2 months after the inoculation of the tumor transplant.

[c] Statistical evaluation in comparison with a control group. NS, Not statistically significant difference.

[d] Two emulsions were prepared; one contained MDP alone and the other contained TDM alone. They were mixed and then injected into the tumors.

[e] An emulsion containing MDP+TDM was frozen at −20°C for 8 days and then thawed and injected into the tumors.

sional injection (Table 1, experiment 3, group 2). The compounds were immunotherapeutically active only when MDP, TDM, and squalane were mixed with each other before being emulsified in saline-Tween solution.

The effect of freezing on the antitumor activity of squalane emulsion containing MDP+TDM was studied next (Table 1, experiment 4). An emulsion containing MDP+TDM was frozen for 8 days at −20°C. This emulsion was immunotherapeutically as active (after thawing) as the freshly prepared emulsion of MDP+TDM in the treatment of 7-day-old tumors.

We had previously studied in guinea pigs the influence of squalane, squalene, hexadecane, and mineral oil on the antitumor activity of BCG cell walls in emulsified form. BCG cell walls were therapeutically active when they were incorporated in any of these four oils (10). In the next experiment (Table 2), we found that squalane and mineral oil, but not squalene or hexadecane, were effective carriers of MDP+TDM.

Finally, we evaluated the minimal dose of MDP+TDM needed to produce tumor regression. Emulsions of MDP+TDM containing various doses of these compounds were prepared. The results presented in Table 3 show that at least 0.05 mg of TDM and 0.01 mg of MDP should be injected intralesionally in emulsified form to cause a statistically significant cure rate.

TABLE 2. *Tumor regression induced in guinea pigs by intralesional injection of MDP+TDM emulsified in different oils*

Material injected[a]		Oil	No. of tumor-free[b] animals/no. tested (at 90 days)	P^c
MDP (mg)	TDM (mg)			
0.06	0.1	Squalane	7/8	=0.001
0.06	0.1	Mineral oil	5/8	<0.05
0.06	0.1	Squalene	2/8	NS
0.06	0.1	Hexadecane	2/8	NS
	0.1	Mineral oil	0/8	
0.1		Mineral oil	0/8	
		Control	0/8	

[a] Emulsions were prepared by ultrasonication and contained 2% oil and 0.2% Tween.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.

TABLE 3. *Minimal intralesional dose of emulsions containing MDP+TDM needed to produce tumor regression in guinea pigs*

Expt	Group	Material injected[a]		No. of tumor-free[b] animals/no. tested (at 90 days)	P^c
		MDP (mg)	TDM (mg)		
1	1	0.25		0/7	
	2	0.25	1	8/8	<0.001
	3	0.25	0.2	8/8	<0.001
	4	0.25	0.04	6/8	<0.01
	5	0.05	0.2	7/8	=0.001
	6	0.05	0.04	7/8	=0.001
	7	0.01	1	7/8	=0.001
	8	0.01	0.2	7/8	=0.001
	9	0.01	0.04	5/7	<0.05
	10		1	0/7	
2	1	0.15	0.05	6/6	<0.001
	2	0.01	0.05	4/7	<0.05
	3	0.01	0.005	0/7	
	4	0.001	0.05	0/7	
	5	0.001	0.005	0/7	
	6[d]	0.01		0/7	
			0.05		

[a] Emulsions were prepared by ultrasonication and contained 2% squalane and 0.2% Tween.
[b] See Table 1, footnote *b*.
[c] See Table 1, footnote *c*.
[d] See Table 1, footnote *d*.

DISCUSSION

The results of the present study indicate that intralesional administration of emulsions containing MDP+TDM was an effective treatment for eradicating dermal tumors and their lymphatic metastases. These results constitute another step forward in the identification of mycobacterial components with antitumor activity. To make an immunotherapeutically effective preparation, MDP and TDM must be mixed together with the oil before emulsification. Similar observations have been reported with emulsions containing TDM and endotoxin (4). McLaughlin et al. reported that one apparent critical function of TDM in the TDM-endotoxin preparation was its ability to enhance the interaction of endotoxin with oil droplets (4). This function of TDM might also be important in emulsions containing MDP+TDM. McLaughlin et al. also reported that only when the TDM and the synthetic analogs of MDP, which they found to be effective, were physically admixed with each other and with the oil were the test materials active (5).

We found that squalane and mineral oil, but not hexadecane or squalene, were effective carriers of these compounds. We have reported that squalene or hexadecane was an effective substitute for mineral oil as a carrier of BCG cell walls in the treatment of guinea pigs with 7-day-old dermal line 10 tumors if the Tween concentra-

tion in the emulsions was 0.002% or less (10). Injectable emulsions containing MDP+TDM cannot be prepared at a Tween concentration of 0.002% or less (unpublished data).

Ribi et al. (7) and McLaughlin et al. (5) have reported that emulsified mixtures of MDP and P_3 (P_3 is highly purified TDM) were not therapeutically active in the guinea pig line 10 model unless an endotoxic component (B_4) was added to the mixture of MDP and P_3 or certain synthetic analogs of MDP were admixed with P_3 (5, 8). Because in our experiments MDP+TDM in emulsified form was highly effective, we asked E. Ribi to send us his MDP, P_3, mineral oil, and solution of 0.2% Tween 80 in phosphate-buffered saline. From his materials we prepared an emulsion containing a mixture of MDP and P_3 (0.3 + 0.1 mg, respectively), 2% mineral oil, and 0.2% Tween 80 (by the ultrasonic method) and injected it into 7-day-old line 10 tumors; 8 out of 10 treated animals were cured without any toxic effect (unpublished data). Ribi et al. have reported that when the mixture of MDP, P_3, and lipopolysaccharide at doses of 0.15 mg each was injected into line 10 tumors, 50% of the animals died within 1 day after treatment, and all surviving animals were lethargic for at least 24 h; of the surviving animals, only 43% were cured (7). We did not see any toxic reaction in our guinea pigs when we used the Ribi reagents or our reagents. The animals received 0.01 to 0.3 mg of MDP plus 0.04 to 1.0 mg of TDM, yet the cure rates were 71 to 100%. Therefore, it seems unlikely that lipopolysaccharide was responsible for the activity of our preparation.

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