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Influence of Type of Oil and Surfactant Concentration on the Efficacy of Emulsified *Mycobacterium bovis* BCG Cell Walls to Induce Tumor Regression in Guinea Pigs

ELIYAHU YARKONI* AND HERBERT J. RAPP

Laboratory of Immunobiology, National Cancer Institute, Bethesda, Maryland 20205

The influence of mineral oil, squalane, squalene, hexadecane, or peanut oil and of the concentration of Tween 80 on the immunotherapeutic capability of emulsified *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) cell walls was studied in guinea pigs, each with an established dermal transplant of a syngeneic hepatocarcinoma and tumor cells in the draining lymph node. Immunotherapy consisted of an intratumoral injection of emulsified cell walls. Conditions were established under which therapeutically effective emulsions could be made with mineral oil, squalane, squalene, or hexadecane. Emulsions made with peanut oil failed to cause tumor regression. Emulsions of squalene or hexadecane were effective substitutes for mineral oil as carriers of cell walls in the absence of added Tween or at a Tween concentration one-hundredth of that used to stabilize the mineral oil-containing emulsions. Cell wall emulsions made with squalane were therapeutically effective over the same range of Tween concentrations used to prepare emulsions containing mineral oil. Cell wall emulsions made without added Tween demonstrated effective antitumor activity even after autoclaving. Emulsions made with Tween separated after autoclaving. Emulsions of whole killed BCG were immunotherapeutically as active as those made with cell walls.

Living *Mycobacterium bovis* BCG has been tested extensively and continues to be tested in clinical trials of cancer immunotherapy. Nonliving BCG preparations have been used to avoid the possibility of systemic infection by the administration of living BCG. Improvement of the efficacy of nonliving mycobacterial preparations for the immunotherapeutic treatment of cancer may require new information on the interactions among components of the emulsified mycobacterial preparations, i.e., mycobacterial fraction(s), oil, and surfactant. The studies reported here were undertaken to investigate the factors affecting the efficacy of mycobacterial components in the treatment of guinea pigs each with an established dermal tumor transplant and microscopic metastasis in the draining lymph node.

MATERIALS AND METHODS

Mycobacterial components and emulsions. BCG cell walls (CW), lots 194 and 278, were obtained from E. Ribi, Rocky Mountain Laboratory, Hamilton, Mont. Heat-killed whole cells of BCG (KC) were purchased from ITR Biomedical Research, University of Illinois at the Medical Center, Chicago. Mineral oil (MO), Drakeol 6VR, was obtained from Penreco, Butler, Pa. Squalane (SQA; $C_{30}H_{62}$) and squalene (SQE; $C_{30}H_{50}$) were obtained from Eastman Kodak Co., Rochester, N.Y. Peanut oil (PNO), USP, was obtained from Welch, Holm, and Clarck Co., Inc., Harrison, N.J. Hexadecane (HD) was obtained from Sigma Chemical Co., St. Louis, Mo. The ultrasonic and grinding methods for preparing injectable emulsions of mycobacterial components have been described previously (16). Briefly, CW (6 mg) were placed in a polypropylene plastic tube. Oil (0.08 ml) was added to the CW, and the mixture was sonicated for 30 s. A 0.5-ml volume of 0.85% NaCl solution containing Tween 80 was added to the mixture, and ultrasonication was applied for 2 to 3 min by submerging the bottom of the tube in the water bath of an ultrasonic cleaner. A 0.4-ml volume of a 0.85% NaCl solution containing Tween was added to the emulsion, and the ultrasonication was continued for 1 to 2 min. NaCl solution (0.85%) containing Tween was added (1.42 ml) to the emulsion. When the CW-oil mixture was emulsified in 0.85% NaCl solution without added Tween, the periods of ultrasonic treatment were doubled. Emulsions containing 0.2% Tween or more were stable, whereas emulsions containing 0.002% Tween or without added Tween contained larger oil droplets that aggregated and floated to the surface of the liquid. For use in immunotherapy, unstable emulsions were mixed well before injecting and the syringe was filled each time with a dose sufficient for only one animal. KC emulsions were prepared similarly. The final concentrations of emulsion components were: CW or KC, 2.5 mg/ml; oil (MO, PNO, SQA, SQE, or HD), 1 to 9%, and Tween 80, zero to 5%. In one experiment we used a mixture of 1 g of SQE with 20 mg of vitamin E (*dl*-α-tocopherol; T 3251, Sigma).

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

Guinea pig tumor line and treatment. Tumor line 10, 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 metastasis in the draining axillary lymph node by 1 week, and death 2 to 3 months after tumor inoculation (9). Animals were treated by intralesional injection of emulsified CW or KC in 0.4-ml volumes 7 to 10 days after tumor implantation, when the average diameter of the skin tumors was 9 to 12 mm. The term "tumor-free animal," as used here, means 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. Differences among groups were evaluated statistically by a 2 × 2 contingency table of Fisher's exact test.

RESULTS

The immunotherapeutic effectiveness of CW emulsions made with SQA, SQE, HD, and PNO was compared to that of emulsions containing MO. Guinea pigs, each with a 7-day-old line 10 tumor, were treated with emulsions of CW containing 3.3% oil and 0.2% Tween. Under these experimental conditions only MO and SQA produced therapeutically effective CW preparations (Table 1). Emulsions of CW containing SQE, HD, or PNO were not therapeutically effective.

Studies with a syngeneic murine fibrosarcoma showed that the number of mice in which tumor regressed completely after intralesional injection of emulsified mycobacterial preparations depended on the concentration of oil in the emulsion (11–13). Therefore, in the next experiment, guinea pigs, each with a 10-day-old dermal tumor, were used to test the efficacy of intralesionally injected CW emulsified in different concentrations of MO, SQA, SQE, or PNO to cause tumor regression (Table 2). Preparations of CW emulsified in 1, 3, or 9% MO or SQA were equally effective in eradication of the skin tumor and the lymph node metastasis. CW emulsified in 9% SQE had moderate antitumor activity; 5 of 11 guinea pigs were cured. The tumor regressive potency of CW emulsions containing 1 or 3% SQE was not significant. CW emulsified in PNO did not produce a significant cure rate when injected intralesionally into 10-day-old tumors.

We have reported that in mice Tween 80 inhibited the antitumor activity of ultrasonically prepared emulsified mycobacterial components when the Tween concentration was 0.9% or more (15). In the next four experiments (Table 3) we tested the effect of Tween concentration on the tumor regression capability of CW emulsified in HD or SQE. The data presented in Table 3 demonstrate that CW emulsified ultrasonically in HD or SQE containing 0.2 or 2.0% Tween had little or no antitumor activity. Intralesionally administered emulsions of CW in HD or SQE

TABLE 1. *Tumor regression in guinea pigs induced by intralesional injection of CW emulsified in different oils*

Expt	Material injected[a]	Type of oil[b]	No. of tumor-free animals/ no. of animals treated (90 days)	P
1	Emulsion	MO	0/6	
		SQA	0/6	
		SQE	0/6	
		HD	0/6	
	CW	MO	6/6	0.001
		SQA	6/6	0.001
		SQE	1/6	NS[c]
		HD	1/6	NS
2	Emulsion	SQA	0/6	
		SQE	0/6	
		PNO	0/6	
	CW	SQA	6/6	0.001
		SQE	1/6	NS
		PNO	0/6	

[a] Control and CW-containing preparations were emulsified ultrasonically and were injected into 7-day-old tumors. CW dose was 1 mg.

[b] Each emulsion contained 3.3% oil and 0.2% Tween 80.

[c] NS, Not statistically significant.

TABLE 2. *Tumor regression induced by intralesional injection of CW emulsified in different concentrations of oils*

Material injected[a]	Type of oil (concn [%])	No. of tumor-free animals/no. of animals treated (90 days)	P
Emulsion	MO (9)	0/10	
	PNO (9)	0/10	
	SQA (9)	0/10	
	SQE (9)	0/9	
CW	MO (9)	11/11	<0.001
	MO (3)	8/10	<0.001
	MO (1)	10/10	<0.001
	PNO (9)	1/10	NS[b]
	PNO (3)	3/10	NS
	PNO (1)	2/10	NS
	SQA (9)	10/11	<0.001
	SQA (3)	10/10	<0.001
	SQA (1)	10/10	<0.001
	SQE (9)	5/11	<0.05
	SQE (3)	2/10	NS
	SQE (1)	3/10	NS

[a] Control and CW-containing preparations were emulsified ultrasonically and were injected into 10-day-old tumors. Each emulsion contained 0.2% Tween 80. CW dose was 1 mg.

[b] NS, Not statistically significant.

TABLE 3. *Tumor regression induced by intralesional injection of CW emulsified in different concentrations of Tween*

Expt	Material injected[a]	Type of oil	Tween concn (%)	No. of tumor-free animals/ no. of animals treated (90 days)	P[b]
1	Emulsion	HD	0.2	0/6	
	CW	HD	0.2	2/6	NS
		HD	0.002	5/6	<0.01
		HD	0	6/6	0.001
		MO	0.2	6/6	0.001
2	Emulsion	HD	2.0	0/10	
	Emulsion	HD	0.2	0/10	
	CW	HD	2.0	0/10	
		HD	0.2	2/10	NS
		HD	0.002	8/10	<0.001
		HD	0	7/10	<0.005
		MO	0.2	6/6	<0.001
3	Emulsion	SQE	2.0	0/7	
	CW	SQE	2.0	3/7	NS
		SQE	0.2	2/7	NS
		SQE	0	7/7	<0.001
		MO	0.2	6/6	<0.001
		MO	0	6/6	<0.001
4	Emulsion	SQE	0.2	0/12	
	CW	SQE	0.2	5/12	<0.05
		SQE	0.002	11/12	<0.001
		SQE	0	12/13	<0.001

[a] Control and CW-containing preparations were emulsified ultrasonically and were injected into 7 (experiment 1), 8 (experiments 2 and 4), or 10 (experiment 3)-day-old tumors. Each emulsion contained 3.3% oil. CW dose was 1 mg throughout.

[b] Cure rates were compared with the emulsion control groups. NS, Not statistically significant.

containing 0.002% or no Tween produced high cure rates.

We have reported that the therapeutic efficacy of emulsified components of mycobacteria in the treatment of a mouse fibrosarcoma was affected by the size distribution of the MO droplets in the emulsion (15). The size distribution of the oil droplets depended on whether the emulsions were prepared by ultrasonication or by mechanical grinding. Emulsions of mycobacterial components prepared by ultrasonication contained smaller oil droplets than those prepared by grinding. Ground emulsion retained antitumor activity over a wider range of Tween concentrations than did ultrasonically prepared emulsions. We have tested the effect of oil droplet size on the antitumor activity of CW emulsified in MO, SQA, SQE, or HD in the experiments summarized in Table 4. From 30 to 40% of the oil droplets in emulsions (5% Tween) prepared by grinding alone were greater than 1 μm in diameter; only about 1% of the oil droplets in emulsions (5% Tween) prepared by or retreated by ultrasonication were larger than 1 μm. The first experiment (Table 4) showed that CW emulsified in MO caused complete regression of the tumor even when the ultrasonically prepared emulsion contained 5% Tween. The second experiment confirmed this observation; similar results were obtained when SQA was used instead of MO. CW emulsified in SQE or HD and 5% Tween were immunotherapeutically inactive regardless of whether they were dispersed by ultrasonication or by grinding.

The effect of freezing on the antitumor activity of MO emulsions of CW was studied in the next experiment (Table 5, experiment 1). CW emulsions containing 0.2% Tween prepared by ultrasonication or by grinding were frozen for 63 days at −20°C. These emulsions were immunotherapeutically as active as freshly prepared emulsions of CW in the treatment of 8-day-old tumors.

Experiments were designed to establish simplified methods for the preparation of immunotherapeutically active emulsions of mycobacterial components that would be suitable for use in the clinic. We tested the therapeutic efficacy of KC because they are less expensive and more

TABLE 4. *Influence of the size distribution of the oil droplets on the antitumor activity of emulsified CW*

Expt	Material injected[a]	Type of oil	Tween concn (%)	Method of emulsification[b]	No. of tumor-free animals/ no. of animals treated (90 days)	P
1	Emulsion	MO	5	G	0/7	
	Emulsion	MO	0.2	US	0/7	
	CW	MO	5	US	6/7	<0.001
		MO	0.2	US	7/7	<0.001
		MO	5	G	7/7	<0.001
		MO	0.2	G	7/7	<0.001
2	Emulsion	MO	5	G	0/6	
		SQE	5	G	0/9	
		SQA	5	G	0/6	
		HD	5	G	0/9	
	CW	MO	5	G	5/6	<0.01
		MO	5	G and US	4/6	<0.05
		SQE	5	G	1/9	NS[c]
		SQE	5	G and US	0/9	
		SQA	5	G	6/6	<0.001
		SQA	5	G and US	6/6	<0.001
		HD	5	G	0/9	
		HD	5	G and US	0/9	

[a] Emulsified preparations each containing 3.3% oil were injected into 8 (experiment 1) or 9 (experiment 2)-day-old tumors. CW dose was 1 mg throughout.

[b] G, Grinding; US, ultrasonication; G and US, emulsions were prepared by grinding and then retreated by ultrasonication.

[c] NS, Not statistically significant.

readily obtainable than CW. We evaluated autoclaving as a means of sterilizing the emulsions. Preliminary experiments showed that autoclaving of emulsions containing 0.2% Tween resulted in separation of most of each preparation into an oil phase and an aqueous phase. CW emulsified without added Tween could be autoclaved without any noticeable change in the appearance of the emulsion. It has been reported that whole killed BCG emulsified with cord factor have antitumor activity (2). We have found that emulsified KC without added cord factor are as effective in the treatment of experimental cancer as CW (14). In the next experiment we tested the effect of autoclaving on the antitumor activity of KC emulsified in different oils without Tween (Table 5, experiment 2). It was found that KC emulsified without added Tween in SQE, SQA, HD, or MO and sterilized by autoclaving caused regression of 9-day-old tumors. When vitamin E was added to SQE to protect the latter from oxidation by air, no change in the antitumor activity was observed. Another way to prepare sterile emulsions is to autoclave dry KC or CW in a polypropylene plastic tube and then to make the emulsion with sterile oil and 0.85% NaCl solution containing 0.2% Tween. Such preparations were immunotherapeutically active (Table 5, experiment 2, last two treatments). When KC emulsified in HD or SQE (without added Tween) were used for immunotherapy, the injected sites healed faster and a smaller scar was left in the skin than when KC emulsified in SQA or MO (with or without Tween) were used.

DISCUSSION

MO is one of the ingredients of emulsified mycobacterial preparations that have been used for treatment of tumors in experimental animals (2, 10, 11) and cancer patients (4, 10). Intralesional injection of mycobacterial components (KC, CW, or methanol extraction residue of BCG) suspended in NaCl solution does not result in eradication of dermal tumor and lymph node metastasis in the guinea pig line 10 model (1, 12). There is some reluctance to use preparations containing MO in humans because of problems associated with other oil-containing vaccines (3, 5, 8). We have reported that SQA, SQE, PNO, and HD were effective substitutes for MO in preparing emulsified mycobacterial components for the treatment of experimental murine fibrosarcoma transplants (13; E. Yarkoni and H. J. Rapp, submitted for publication). SQE is an intermediate product in the biosynthesis of cholesterol and can be found in various tissues of mammals and in some plants. SQA is obtained by complete hydrogenation of SQE. HD is one of the components of MO, but unlike MO it has been found to be digestible in various tissues

TABLE 5. *Tumor regression induced by intralesional injection of CW preparations after freezing or autoclaving*

Expt	Material injected[a]	Type of oil (concn [%])	Tween concn (%)	Prepn method[b]	No. of tumor-free animals/no. of animals treated (90 days)	*P*
1	No treatment				0/10	
	CW	MO (3.3)	0.2	US, Fresh	7/10	<0.005
		MO (3.3)	0.2	G, Fresh	10/10	<0.001
		MO (3.3)	0.2	US, Frozen	10/10	<0.001
		MO (3.3)	0.2	G, Frozen	9/10	<0.001
2	No treatment				0/8	
	Emulsion	SQE + vitamin E[c] (3.3)	0	US, Fresh	0/8	
	KC	SQE + vitamin E (3.3)	0	US, Fresh	5/5	0.001
	KC	SQE (3.3)	0	US, Fresh	8/8	<0.001
		SQE + vitamin E (3.3)	0	US, A-A	8/8	<0.001
		SQA (3.3)	0	US, A-A	8/8	<0.001
		HD (3.3)	0	US, A-A	7/8	0.001
		MO (3.3)	0	US, A-A	8/8	<0.001
		MO (3.3)	0.2	US, A-B	8/8	<0.001
		MO (3.3)	0.2	US, A-B	4/5	<0.01

[a] Dose of either KC or CW was 1 mg throughout.

[b] US, Ultrasonication; G, grinding; Fresh, emulsion was injected within 2 h after preparation; Frozen, emulsion was frozen (−20°C) for 63 days before inoculation; A-A, emulsion was autoclaved after emulsification; A-B, the dried KC or CW were autoclaved in the tube before emulsification.

[c] The SQE contained vitamin E, 20 mg/g.

and among widely divergent animal species (7). SQE, SQA, and HD have additional advantages over MO; their structures are known, they can be synthesized in vitro, and each of them contains only one component, unlike MO which contains thousands of compounds.

The studies reported here demonstrated that SQA, SQE, and HD but not PNO were effective substitutes for MO as carriers of CW or KC in the treatment of guinea pigs with a transplanted hepatoma. SQA and MO were effective carriers for CW or KC over a wider range of Tween concentrations than were SQE or HD. The fact that SQE and HD are digestible and SQA and MO are not may account for this difference. CW emulsified in SQE or HD had antitumor activity when the Tween concentration was 0.002% or less, but not 0.2% or more. Perhaps, at sufficiently high concentrations, Tween promotes the digestion of SQE and HD. PNO was not tested with concentrations of Tween in the range of 0.002% or less because under that condition the oil did not become emulsified even after 1 h of ultrasonic treatment.

Studies with a murine fibrosarcoma model showed that the size distribution of the mineral oil droplets affects the antitumor activity of emulsified mycobacterial components (15). The size distribution of the oil droplets depends on whether the emulsions are prepared by ultrasonication or by grinding. Emulsions prepared by ultrasonication contain smaller oil droplets than do those prepared by grinding. CW emulsified in MO by grinding in a salt solution containing 4.5% Tween retained antitumor activity, whereas ultrasonically prepared emulsions containing 4.5% Tween were immunotherapeutically inactive (15). In the guinea pig model, ground as well as ultrasonically prepared emulsions of CW in MO or SQA containing 5% Tween were immunotherapeutically active. CW emulsified by grinding or by ultrasonication in SQE or HD with 5% Tween did not cause regression of the line 10 tumors. The conditions under which emulsified CW could cause regression of tumors in mice and guinea pigs are summarized in Table 6.

TABLE 6. *Influence of type of oil and Tween concentration on the efficacy of tumor regression in mice and guinea pigs by emulsified CW*

Type of oil	Tween concn (%)	Emulsification method[a]	Tumor regressive efficacy[b]	
			Mice	Guinea pigs
MO	0	US	±	+
	0.2	US or G	+	+
	1-5	US	−	+
	5	G	+	+
SQA	0	US	±	+
	0.2	US	+	+
	5	US	−	+
	5	G	+	+
SQE	0	US	±	+
	0.2	US	+	−
	2-5	US	−	−
	5	G	+	−
HD	0	US	+	+
	0.2	US	+	−
	5	US	−	−
	5	G	+	−
PNO	0.2	US	−	−

[a] US, ultrasonication; G, grinding.

[b] Mice: Emulsified CW (0.6 mg/0.1 ml per mouse; 9 or 10% oil) were injected intralesionally into 5-day-old syngeneic fibrosarcoma transplants, about 4 mm in diameter. Guinea pigs: Emulsified CW (1 mg/0.4 ml per guinea pig; 3.3% oil) were injected intralesionally into 7- to 10-day-old line 10 tumors, 9 to 12 mm in diameter. +, Cure rates were statistically significant in comparison with the control groups ($P < 0.01$); ±, cure rates in comparison with the control groups were not always statistically significant ($P < 0.05$ or $P > 0.05$); −, cure rates were not statistically significant in comparison with the control group ($P > 0.05$).

Emulsions of CW containing 0.2% Tween that were thawed after having been frozen at −20°C for several months remained stable in appearance and immunotherapeutically as active as freshly prepared emulsions. Autoclaving of CW emulsions containing 0.2% Tween resulted in separation of the emulsions into oil and aqueous phases. CW emulsions made without adding Tween retained their emulsion form and antitumor activity after autoclaving. Stable emulsions of CW or KC containing 0.2% Tween could be prepared using heat sterilization by first autoclaving the mycobacterial component as a dry powder in a polypropylene tube and then adding the remainder of the ingredients to be emulsified in sterile form. When dry CW were autoclaved they could not be incorporated into the oil droplets unless 0.2% Tween was present. In contrast, dry KC after autoclaving could be emulsified in 0.85% NaCl solution without added Tween.

This investigation demonstrates that the conditions required for producing emulsions of mycobacterial components with optimal antitumor activity in guinea pigs with a transplanted hepatoma sometimes are not the same as those in mice with a transplanted fibrosarcoma. Nevertheless, mycobacterial components emulsified in MO and 0.2% Tween were effective in producing

tumor regression in mice, guinea pigs, cows, and humans (2, 4, 6, 10–16). We have found that intralesional administration of CW or KC emulsified in HD or SQE without added Tween led to eradication of syngeneic tumor transplants in the skin and of micrometastasis in the draining lymph nodes of guinea pigs. In addition, the injected sites healed faster and a smaller scar was left than when MO was used. Clinical trials of the efficacy of emulsified mycobacterial components in the treatment of humans with cancer might be facilitated by the substitution of an oil such as SQE or HD for MO in the preparation of the adjuvant.

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