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Histopathology of Tumor Regression by Cord Factor, Turpentine or Endotoxin, Dissociation of Therapy and Granuloma Formation

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Abstract—*Histologic changes and therapeutic effects seen after a single injection of emulsified trehalose-6,6′-dimycolate, a mycobacterial glycolipid, into growing dermal implants of a mouse fibrosarcoma were similar to those seen after injection of endotoxin and of emulsified turpentine. Intralesional injection of any of these agents caused the tumors to regress in a significant number of animals. The number of tumor free mice capable of resisting the growth of tumor cells in a challenge inoculum, however, was greater for animals treated with trehalose-6,6′-dimycolate than those treated with turpentine or endotoxin. Granuloma formation was not a requirement for tumor regression in this mouse model.*

INTRODUCTION

INOCULATION of trehalose-6,6′-dimycolate (TDM), a mycobacterial glycolipid known also as cord factor, into animals produces some of the biological effects of mycobacterial infection [1]. One of these effects is the formation of granulomas [2–4]. It has been postulated that granuloma formation may be required for tumor regression induced by living BCG organisms [5, 6] or by emulsified TDM [7]. Recently we have reported that TDM was able to cause regression of a growing transplanted syngeneic fibrosarcoma in the skin of mice [8]. The studies reported here were undertaken to determine whether the regression of the fibrosarcoma was related to granuloma formation by emulsified TDM. In addition, the tumor regressive potency and the types of cellular reactivity induced by TDM were compared with those induced by turpentine and by endotoxin.

MATERIALS AND METHODS

Animals

Male C3H/HeN mice 18–20g were obtained from the Laboratory Aids Branch, Division of Research Services, National Institutes of Health (Bethesda, Md). Animals were housed in groups of 6 in plastic cages and fed Wayne Lab Blox and tap water *ad libitum*.

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Tumor

Tumor 1023 arose in the subcutaneous tissues of a C3H/HeNIcr male mouse after implantation of paraffin pellets containing 1% 3-methylcholanthrene. The tumor was maintained by serial trocar passage in syngeneic immunodepressed mice as described previously [9]. Transplant generations 10–12 were used in these experiments. Tumor cell suspensions were prepared by pronase–DNase digestion of minced tumor tissue [9]. More than 95% of the tumor cells excluded 0.1% trypan blue.

Treatment of mice with established tumor transplants

Mice (in groups of 27) each received 1×10^6 viable 1023 tumor cells intradermally on their dorsal surface in 0.05 ml Hanks balanced salt solution. At 6 days thereafter, each group of mice was treated by the intratumoral injection of 0.05 ml of one of the following agents: TDM (0.15 mg) incorporated in emulsified mineral oil droplets; emulsified turpentine (10% or 1%); endotoxin (0.3 or 0.1 mg) in 0.15 M NaCl solution;

mineral oil in water emulsion. Fifteen mice from each treatment group were used to determine the efficacy of tumor regression and the remainder were used for histopathology.

Evaluation of tumor growth

Tumor incidence was determined by weekly observation for 2 months. Differences in tumor incidence among groups were evaluated statistically by the Wilcoxon non-parametric rank test [10].

Detection of tumor-specific immunity

Treated mice that remained tumor-free were rechallenged with 1×10^6 1023 tumor cells intradermally on their ventral surface. Injection sites were observed for 30 days thereafter for growth of rechallenge inoculum.

BACTERIAL FRACTIONS AND EMULSIONS

Endotoxin

Endotoxin from the heptoseless (Re) mutant of *Salmonella typhimurium* strain G30/C21, was obtained from Hamilton Research Laboratory, Hamilton, Montana 59840. Endotoxin was dissolved in 0.15 M NaCl solution for intralesional injection.

Trehalose-6,6′-dimycolate (TDM) emulsion

TDM from *Mycobacterium bovis*, substrain AN5, was obtained from Dr. E. Lederer, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France. TDM (3 mg) and mineral oil (Drakeol 6VR, 0.1 ml) were placed in a polypropylene plastic tube (12 × 75 mm, Falcon, Oxnard, CA) and the mixture heated at 56°C for 1 min to dissolve TDM in oil. Half of a millilitre of 0.15M NaCl solution containing 0.2% Tween 80 was added and ultrasonication (ultrasonic cleaner, Model CC-.25, Beckman Instruments, Inc., Fullerton, CA) was applied for 2 min. Four tenths of a millilitre of 0.15M NaCl solution containing 0.2% Tween 80 was added to the emulsion. Final concentration of each emulsion component was: 3 mg/ml TDM, 10% mineral oil, and about 0.2% Tween 80. Emulsion control without TDM was prepared similarly.

Turpentine emulsion

Rectified turpentine (Permanent Pigments, Inc., Cincinnati, OH) was emulsified in 0.15M NaCl solution containing 0.2% Tween 80 by ultransonic treatment. Final concentration of each emulsion component was: turpentine, 1 or 10% (by volume) and about 0.2% Tween 80.

Histopathology

Two or three animals from each treatment were killed at 3, 7, 14 and 21 days. The injected sites and the superficial inguinal lymph nodes were removed and fixed in Bouin's fluid and stained with hematoxylin and eosin.

RESULTS

Immunotherapy

Mice (in groups of 15) each received an intradermal inoculation of 1×10^6 tumor cells. Six days after inoculation, tumors ranged from 3 to 4 mm in diameter. Tumors were treated by intralesional injection of 0.05 ml of one of the following preparations: emulsion alone (10% oil), emulsified TDM (0.15 mg/0.05 ml), emulsified turpentine (10 or 1%), endotoxin (0.3 or 0.1 mg/0.05 ml). After 60 days the following proportions of animals treated by the indicated agent were tumor-free: emulsion alone—0/15; TDM—14/15; 10% turpentine—13/15; 1% turpentine—2/15; 0.3 mg endotoxin—11/15; 0.1 mg endotoxin—4/15 (Table 1). Twelve of the 14 animals rendered tumor-free by TDM resisted the growth of a tumor cell challenge (Table 1). Turpentine and endotoxin were less effective than TDM in promoting tumor specific rejection immunity.

Histopathology

Effect of mineral oil emulsion. Three days after the injection of mineral oil emulsion edema and a modest inflammatory exudate with prevalence of mononuclear phagocytic cells in the dermis and subcutaneous tissue were evident. Tumor cells appeared to be undamaged (Fig.1a). After 7–21 days the modest inflammatory reaction subsided and consisted of only a few vacuolated macrophages surrounding the progressively growing tumor.

Effect of emulsified TDM. Three days after intralesional injection the skin overlaying the tumor became ulcerated. Areas of edema, hemorrhage and coagulative necrosis were present in the dermis and in the subcutaneous tissue. A massive acute inflammatory reaction with a marked predominance of polymorphonuclear leukocytes (PMNL) resulted in areas of colliquative necrosis (Fig. 1b). Tumor cells appeared to be damaged and degenerating. Seven to twenty-one days after TDM

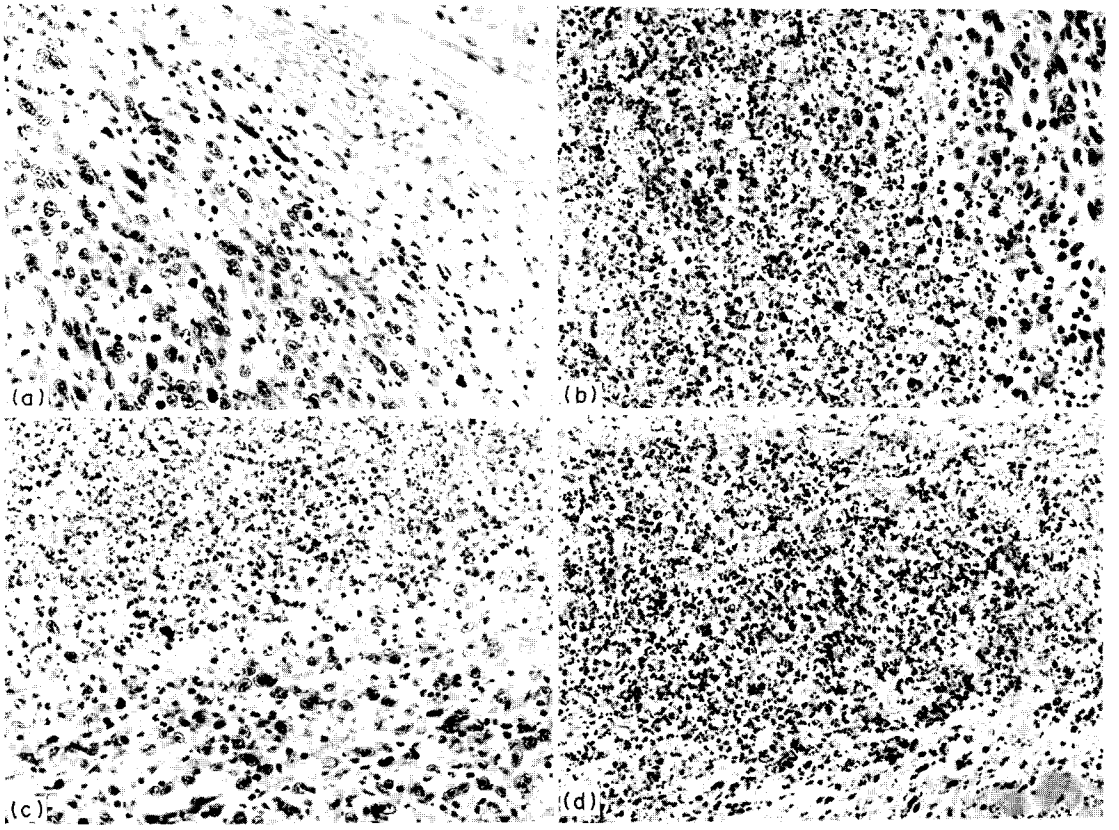

Fig. 1. *Histologic changes at the tumor site* 3 days *after intratumoral injection of:* a—*emulsion* (10% oil); b—*TDM* (0.15 mg); c—*turpentine* (10%); d—*endotosin* (0.3 mg). (×350).

Table 1. Tumor regression by endotoxin, emulsified TDM or emulsified turpentine

Material injected	Dose*	Number tumor-free animals/number animals treated	*P*	Number immunized animals/number tumor-free animals
No treatment	—	0/15	—	—
Mineral oil emulsion	10%	0/15		—
TDM	0.15mg	14/15	<0.001	12/14
Turpentine	1%	2/15	NS†	0/2
	10%	13/15	<0.001	3/13
Endotoxin	0.1 mg	4/15	NS	2/4
	0.3 mg	11/15	<0.001	4/11

*Volumes injected into tumors were 0.05 ml.
†NS, not statistically significant.

administration mononuclear cell infiltration and initial reparative phenomena were observed. No tumor cells or granulomas were detected at the tumor site or in the draining lymph nodes.

Effect of emulsified turpentine. Three days after the injection of 10% turpentine necrosis and hemorrhagic areas were seen in the dermis, the subcutaneous tissue and the muscular stratum. An acute predominantly PMNL inflammatory infiltration was evident. Areas of tumor necrosis surrounded by normally dividing tumor cells were seen (Fig.1c). Seven days after treatment tumors regressed completetly in most of the treated animals and initial organization of the necrotic area was observed. After another 7–14 days the lesion was completely repaired and substituted with fibrous tissue. No tumor cells were seen.

After the inoculation of 1% turpentine, tissue damage at the injected site and initial tumor necrosis were less evident than that seen after inoculation of 10% turpentine.

Effect of endotoxin. Three days after intralesional injection of 0.3 mg of endotoxin, areas of coagulative and colliquative necrosis in the dermis and in the subcutaneous tissue were present. Marked PMNL infiltration was evident and degenerating tumor cells could be seen (Fig. 1d). At 7–21 days after endotoxin administration no tumor cells could be detected. Presence of lymphomononuclear infiltrate and granulation tissue were noted.

After the injection of 0.1 mg of endotoxin the reaction at the injected site was analogous to the reaction induced by 0.3 mg, but less pronounced. In most of the histologic preparations from animals treated with 0.1 mg of endotoxin tumor cells were normally dividing.

No tumor metastasis was detected in the draining lymph nodes in any of the mice examined in this study.

DISCUSSION

The results of the present study indicate that intralesional administration of emulsified TDM causes regression of established syngeneic tumors in C3H/HeN mice, without any evidence of granuloma formation at the injected site or in the draining lymph nodes. Bekierkunst *et al.* reported that 0.01 mg of emulsified TDM, injected into the footpad of Swiss mice induced granuloma formation in the injected site and in the draining popliteal lymph node, 15 and 5 days, respectively, after TDM administration [3]. Our failure to find granulomas at sites of TDM inoculation may be due to variation among mouse strains in their response to TDM injection. This possibility is supported by the results of a study in which different inbred strains of mice reacted in different ways to injections of emulsified TDM [3] or emulsified killed BCG [11].

The histological changes produced by 10% turpentine were similar to those induced by TDM, but TDM was better than turpentine as a promoter of tumor specific immunity. Twelve of fourteen mice in which complete tumor regression occured, after intratumoral injection of emulsified TDM, resisted the growth of tumor cells in a challenge inoculum. In contrast, only 3 out of 13 mice in which complete tumor regression occured, after injecting turpentine, were able to reject the challenge. It has been reported that TDM enhanced the immune response to antigens [12–14]. Induction of chemotaxis [15] and stimulation of macrophages by TDM [16] may contribute to the antitumor activity of TDM. Direct cytotoxicity by TDM or endotoxin was not seen *in vitro*. Addition of 0.05 ml of endotoxin (0.3 mg) or emulsified (10% oil) TDM (0.15 mg) into 1 ml medium of

tumor cell (No. 1023) culture, did not produce cytotoxicity during the 24 hr incubation. In contrast, addition of 0.05 ml of 1% turpentine to a culture of tumor cells resulted in complete destruction of the culture in less than 24 hr (E. Yarkoni—unpublished data).

In contrast to the results presented in this study, Hanna *et al.* reported that intralesional injection of turpentine enhanced tumor growth [6]. Whether these contradictory observations are due to methodological differences or to differences in responses of mice and guinea pigs to turpentine is not known. The latter possibility may be more likely since differences between these species have been shown for the antitumor effect of TDM and endotoxin. A transplantable hepatocarcinoma of guinea pigs has been used to study the antitumor activity of TDM. Emulsified TDM combined with ET induced regression of guinea pig tumors; emulsified TDM or ET alone, however, were inactive [17]. In contrast, emulsified TDM or ET alone were both found to be effective, in this study, in regression of an established murine tumor. Intralesional injection of emulsified TDM was not able to cause regression of 7-day-old hepatocarcinoma transplants in the skin of the guinea pigs, although it was able to induce granuloma formation in the skin or lungs of guinea pigs after intradermal or intravenous administration, respectively [4, 18].

The present study showed that emulsified TDM was effective in regression of established murine tumor without the induction of granuloma formation in the injected site and in the draining lymph nodes. It is concluded that certain agents administered intralesionally may cause tumor regression without granuloma formation.

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