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Regression of Tumors in Guinea Pigs After Treatment with Synthetic Muramyl Dipeptides and Trehalose Dimycolate

Abstract. *A high incidence of tumor regression was observed in guinea pigs bearing transplantable, line-10 hepatocellular carcinomas when synthetic muramyl dipeptides combined with trehalose dimycolate in oil-in-water emulsions were injected directly into the tumors. These compounds are promising candidates to replace viable bacillus Calmette-Guérin in cancer immunotherapy in humans and animals.*

Approximately 8 years ago, a report (*1*) appeared describing the use of viable *Mycobacterium bovis*, strain bacillus Calmette-Guérin (BCG), to treat dermal and metastatic tumors in guinea pigs. This model system subsequently proved useful for evaluating immunopotentiating substances with antitumor activity for use in the treatment of human cancer (*2-4*). Here we describe the use of synthetic, water-soluble muramyl dipeptides (also termed adjuvant dipeptides) in combination with trehalose dimycolate (isolated from mycobacteria) to replace viable mycobacteria in this immunotherapy model system.

Tumors in humans (*5, 6*) and animals (*2*) have been treated by injecting live BCG directly into the growing tumors. However, such treatment may produce slowly healing ulcers, protracted fever, hepatitis, and occasional anaphylactic reactions (*2, 5-8*). Because of these undesirable and sometimes life-threatening side effects, it has been a goal of many investigators to replace viable BCG with defined, nontoxic components isolated from mycobacteria. Such replacement would also assist investigators to study the biological processes of tumor regression after immunotherapy. Considerable progress was made in this direction when Ribi and co-workers found that oil-in-water emulsions containing either cell walls of mycobacteria (*9*) or cell wall skeletons combined with trehalose dimycolate (*10*) were effective in curing tumors in guinea pigs. These nonviable preparations have been used successfully in clinical trials for treatment of spontaneous tumors in humans (*3*) and animals (*11*).

The component of mycobacterial cell wall preparations that is responsible for tumor regression is not known, but the synthetic compound *N*-acetylmuramyl-L-alanyl-D-isoglutamine represented a reasonable candidate. This unit was found to be the minimal structural entity that can replace mycobacterial whole cells in Freund's complete adjuvant and permit antibody stimulation and delayed hypersensitivity reactions to a variety of antigens (*12*). Covalently lipid-bound muramyl dipeptides suppressed growth of tumors in mice (*13*) and promoted regression of tumors in guinea pigs (*4*). On the basis of these observations and with the hope of finding a potent muramyl dipeptide, we investigated the antitumor activity of certain synthetic analogs of *N*-acetylmuramyl-L-alanyl-D-isoglutamine.

Ten different muramyl dipeptides (150 μg of each) (*14*) were tested alone and in combination with trehalose dimycolate (150 μg). Test materials were admixed in mineral oil and then suspended in 0.2 percent Tween 80 in phosphate-buffered saline to form oil-in-water emulsions (*15*). The final concentration of oil was 0.75 percent by volume. Test emulsions (0.4 ml) were injected directly into established, line-10 hepatocellular carcinomas 6 days after intradermal transplantation of 10^6 tumor cells. The tumors were 8 to 11 mm in diameter and had metastasized to regional draining lymph nodes (*2*). Surviving animals received another transplantation of 10^6 line-10 cells 3 months after treatment and were observed for an additional month. All of the animals rejected this challenge.

In the absence of trehalose dimycolate, none of the muramyl dipeptides caused regression of tumors in treated animals (*16*). Trehalose dimycolate was inactive when tested alone. The tumor-regressive activity of the muramyl dipeptides combined with trehalose dimycolate was greatest for *N*-acetyl-4,6-di-*O*-octanoylmuramyl-L-valyl-D-isoglutamine and *N*-acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine (Table 1). Subsequent dose-response studies indicated that the latter is the more effective compound on a per microgram basis (*17*). Although all of the tested muramyl dipeptides except *N*-acetylmuramyl-D-alanyl-D-isoglutamine showed adjuvant activity (*18*), three of these compounds in combination with trehalose dimycolate did not have significant antitumor activity. Substitution of an *N*-glycolyldesmethylmuramic acid moiety for an *N*-acetylmuramic acid group or substitution of L-serine, L-valine, or L-α-aminobutyric acid for the L-alanyl moiety significantly increased the antitumor activity of the muramyl dipeptides combined with trehalose dimycolate.

Table 1. Tumor regression after treatment. Values are numbers of guinea pigs cured of dermal and metastatic tumors over numbers of animals treated. Data shown are pooled from two separate experiments. No cures were observed in animals treated with any muramyl dipeptide in the absence of trehalose dimycolate (*16*).

Synthetic muramyl dipeptide (150 μg) tested with trehalose dimycolate (150 μg)	Observed tumor regression
N-Acetylmuramyl-L-alanyl-D-isoglutamine	1/17
N-Acetylmuramyl-D-alanyl-D-isoglutamine	0/9
N-Acetyl-4,6-di-*O*-octanoylmuramyl-L-alanyl-D-isoglutamine	1/9
N-Acetyldesmethylmuramyl-L-alanyl-D-isoglutamine*	2/19
N-Acetylmuramyl-L-threonyl-D-isoglutamine	3/9
N-Acetylmuramyl-L-seryl-D-isoglutamine	10/17†
N-Acetyldesmethylmuramyl-L-valyl-D-isoglutamine*	10/17†
N-Glycolyldesmethylmuramyl-L-alanyl-D-isoglutamine	7/9†
N-Acetyl-4,6-di-*O*-octanoylmuramyl-L-valyl-D-isoglutamine	16/18†
N-Acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine*	17/18†
Trehalose dimycolate alone (control)	0/17
Emulsion of oil, Tween 80, and phosphate-buffered saline (control)	0/14

*These are trivial names for 2-acetamido-2-deoxy-D-gluco-3-*O*-yl-acetyl dipeptides. †Significantly different from the value for trehalose dimycolate-treated controls (*21*) (chi-squared, 2 × 2 table analyses, $P \leq .05$).

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Direct interaction of the muramyl dipeptides with trehalose dimycolate in the therapeutic emulsions was essential for tumor regression. Two emulsions that were prepared separately, one containing trehalose dimycolate and one containing *N*-acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine, and then mixed and injected into tumors did not produce significant tumor regression compared to controls (*19*). Only when the trehalose dimycolate and muramyl dipeptides were physically admixed with each other and with the oil, before addition of the aqueous solution of the emulsions (as for the experiments in Table 1), were the test materials active. This finding is similar to the results of a study of the physical interaction of trehalose dimycolate and endotoxic glycolipids (*20*). In that case it was proposed that water-soluble substances (such as the muramyl dipeptides in this study) complexed with the trehalose dimycolate through the trehalose portion of the molecule; the hydrophobic mycolic acid residues were envisioned buried in the oil droplets of the emulsions (*20*). These results indicate that a tertiary complex of muramyl dipeptide, trehalose dimycolate, and oil droplets is required for therapeutic effectiveness in the present system.

The synergistic antitumor effect of certain muramyl dipeptides and trehalose dimycolate described here may be useful for treating cancer in humans and animals. It must first be shown that these immunopotentiating agents can be used safely in humans and animals suffering from the stresses of cancer and its associated disease conditions.

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17. C. A. McLaughlin, S. M. Schwartzman, G. H. Jones, unpublished results. Ten of 15 treated animals were cured following injection of 1.5 μg of *N*-acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine combined with 150 μg of trehalose dimycolate, whereas only one of nine treated animals were cured following injection of 1.5 μg of *N*-acetyl-4,6-di-*O*-octanoylmuramyl-L-valyl-D-isoglutamine combined with 150 μg of trehalose dimycolate.
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19. C. A. McLaughlin, S. M. Schwartzman, G. H. Jones, unpublished results. Lyophilized *N*-acetyldesmethylmuramyl-L-α-aminobutyryl-D-isoglutamine was mixed with mineral oil and then suspended as an emulsion in Tween in saline. Trehalose dimycolate was mixed with mineral oil and suspended in a separate emulsion. The two emulsions were combined and 0.4 ml containing 150 μg each of muramyl dipeptide and trehalose dimycolate was injected into tumors of guinea pigs. One of nine treated animals was cured. None of the control animals receiving trehalose dimycolate or oil-in-water emulsions (nine animals per group) were cured.
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Visual Aftereffects Derived from Inspection of Orthogonally Moving Patterns

Abstract. *Alternate inspection of patterns moving in orthogonal directions induces an aftereffect in which a stationary test pattern seems to move in a new direction. This direction is the resultant of the two directions of aftereffect that would have arisen from separately inspecting each of the moving patterns. The direction in which objects appear to move, like their color and depth, can thus depend on a synthesis of unperceived components.*

When a pattern observed for a minute or so while moving is stopped, it appears to move for some time in the opposite direction (*1, 2*). This visual movement aftereffect (MAE) may be induced by linear, rotational, or radial (expanding-contracting) movement. It varies in strength as a function of numerous stimulus variables, including the direction of inducing movement and the luminance and contrast relationships of the moving and stationary patterns (*3*). The MAE has been interpreted in terms of an imbalance in the activity of neural units responsive to opposite directions of movement; units tuned to one direction are claimed to adapt negatively during prolonged observation, so when movement ceases, those tuned to the opposite direction are briefly more active (*4*).

In an experiment originally designed to test the hypothesis that the direction of the MAE is contingent on the orientation of bars in a moving grating, we followed the general procedure first described by McCollough (*5*) for generating a contingent aftereffect. Subjects inspected 45° bars moving continuously toward 135° [within a ring of anchoring dots (*6*)] in alternation with 135° bars moving toward 225° (Fig. 1A). The duration of each phase was 10 seconds. After 10 to 30 minutes of these alternating inspections, we replaced the moving grating with a stationary test pattern consisting half of 45° and half of 135° bars (Fig. 1A). We expected that, if the direction of the MAE were contingent on line orientation, the two sets of bars would appear to move orthogonally to one another, each in a direction opposite to that of the corresponding bars during inspection. None of the bars did so. Instead, all bars appeared to move together

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