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Stimulation of Chemiluminescence and Resistance Against Aerogenic Influenza Virus Infection by Synthetic Muramyl Dipeptide Combined with Trehalose Dimycolate

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The effect on respiratory burst of splenic cells from mice pretreated with oil-in-water emulsions of muramyl dipeptide (MDP), trehalose dimycolate (TDM), or the combination of MDP with TDM was studied by luminol-dependent chemiluminescence in response to stimulation by zymosan. Spleen cells from mice pretreated with TDM, but not those of mice treated with MDP, generated increased chemiluminescence. Spleen cells from animals pretreated with the combination of MDP and TDM exhibited markedly enhanced chemiluminescence activity. The effect of enhanced activity of preparations containing MDP combined with TDM was further examined in vivo by an aerosol infection of pretreated mice with a mouse-pathogenic influenza virus. Pretreatment with 6-O-acyl analogs and one ubiquinone derivative of MDP alone did not induce any resistance against influenza virus. Significant protection was conferred only when MDP and certain analogs were combined with TDM. The enhancement of nonspecific resistance to influenza virus infection was related to the chemical structure of the synthetic immunostimulant. A greater degree of protection was induced by the combination of TDM with the lipophilic derivatives like B 30-MDP and L-18 MDP.

Adjuvants of bacterial origin are widely used as immunostimulants to enhance a variety of host immune responses. Detailed investigations on mycobacteria, corynebacteria, and nocardiae have led to the identification of their cell wall structure as a mycolic acid-arabinogalactan-mucopeptide complex which contains adjuvant-active moieties (2). The minimum structure essential for bacterial adjuvanticity is a peptidoglycan monomer, *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (7). In addition to this glycopeptide, other immunopotentiating components present on the cell walls are glycolipids like 6,6′-diesters of trehalose (14). Defined microbial components, like muramyl dipeptide (MDP) and trehalose dimycolate (TDM; cord factor), have been synthesized and have attracted much attention because of their antitumor, adjuvant, and resistance-stimulating activities (15, 24).

Macrophages appear to be among the target cells for the immunopotentiating activity of MDP and TDM. Recent studies have demonstrated that MDP can inhibit macrophage migration (21); enhance the production of T-cell-activating monokines (10), collagenase and prostaglandins (28), superoxide anion (23), and chemiluminescence (CL) (17); and augment the cytolytic activity of macrophages (25). TDM has been shown to stimulate macrophage lysosomal enzyme, acid phosphatase, phagocytosis (30), and chemotaxis (11); induce the release of monokine (26) and hydrogen peroxide (16); and enhance cytotoxic activity against mastocytoma cells (15). The stimulation of oxidative metabolism in phagocytic cells results in an increased hexose monophosphate shunt activity and the generation of activated oxygen metabolites like singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical with the emission of photons (1). The release of energy in the form of light, termed CL, can be amplified by the cyclic hydrazide luminol (6).

The recent studies have demonstrated that in certain experimental tumor and microbial models in which MDP or TDM alone is ineffective, a combination of these two compounds in an oil-in-water emulsion results in a preparation which can induce a high degree of immunopotentiation (3, 18, 20, 29). Only a few in vitro studies have been performed with the MDP-plus-TDM preparations. In the present study, we investigated the effect on respiratory burst of splenic cells from mice preteated in vivo with MDP, TDM, or the combination MDP plus TDM, using a luminol-dependent CL assay.

MATERIALS AND METHODS

Animals. BALB/c mice 5 to 6 weeks old were purchased from Zentralinstitut für Versuchstiere, Hanover, Federal Republic of Germany, and NMRI mice were obtained from the pathogen-free colony maintained by the Bundesgesundheitsamt, Berlin, Federal Republic of Germany.

Immunomodulators. Seven different synthetic glycopeptides were used in these studies. *N*-Acetyl muramyl-L-alanyl-D-isoglutamine was purchased from Institut Pasteur Production, Paris, France. 6-*O*-(2-Tetradecylhexadecanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (B30-MDP), 6-*O*-(3-hydroxy-2-docosylhexacosanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (BH48-MDP), 6-*O*-octanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (L8-MDP), 6-*O*-stearoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (L18-MDP), 6-*O*-isopentadecanoly-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (Iso.15-MDP), and 2,3-dimethoxy-5-methyl-6-(9′-carboxynonyl)-1,4-benzoquinone-*N*-acetyl muramyl-L-valyl-D-isoglutamine-methyl ester (QS-10-MDP-66) were synthesized by previously described methods (12, 13). TDM was extracted from free lipids of *Mycobacterium tuberculosis* Aoyama B and was purified by pressure elution through columns of microparticulate silica gel by the Ribi Immunochemical Research Inc., Hamilton, Mont.

Pretreatment. MDP or TDM and a small amount of squalane (C30 H62) (Eastman Kodak Co., Rochester, N.Y.),

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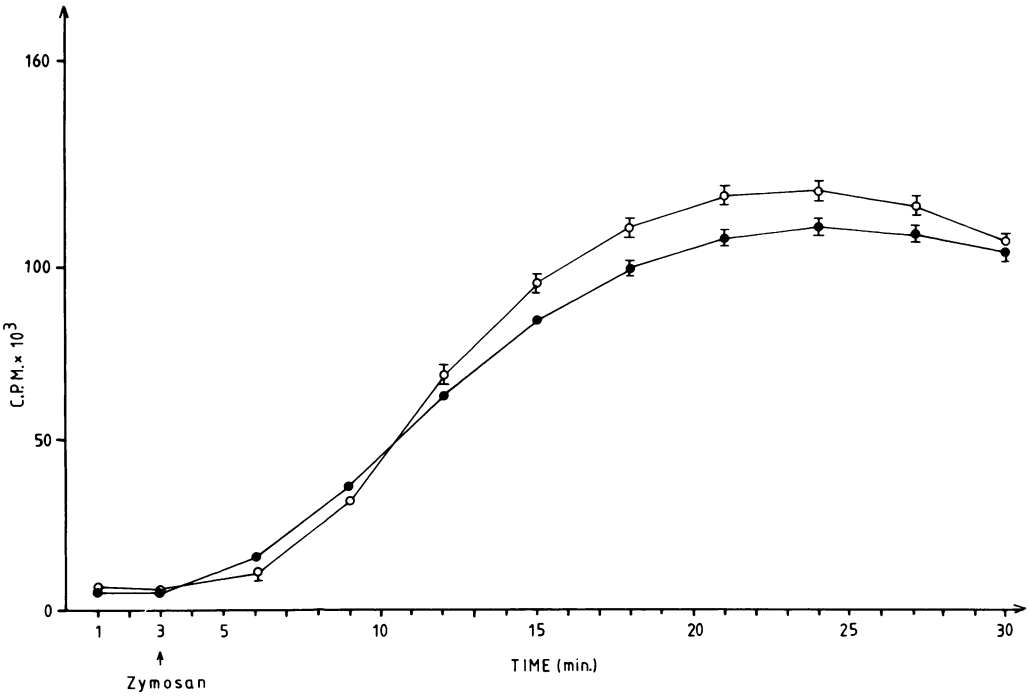

FIG. 1. Zymosan-induced CL response of spleen cells from mice pretreated with oil-in-water emulsion of MDP (○) and from control mice (●). Each point represents mean value ± standard error of three experiments.

a perhydrogenated form of the metabolizable oil squalene, were blended into a smooth paste with a Teflon pestle in a tissue grinder and then were mixed with buffered saline containing 0.02% Tween 80 until a well-dispersed emulsion was obtained. The final concentration of oil was about 1%. The preparations containing the combination of MDP and TDM were prepared by dissolving TDM in chloroform-methanol (95:5) and adding the required amount of MDP to it. The organic solvent was evaporated by a gentle jet of N_2 gas, and the combined product was dried overnight in a desiccator containing $CaCl_2$.

The oil-in-water emulsions containing MDP, TDM, the MDP-plus-TDM combination, and the oil-in-Tween saline (vehicle control) were administered to groups of 10 to 20 animals by the intravenous route (0.2 ml) into the tail vein. Aerosol infection with mouse-pathogenic influenza virus, strain A/PR/8/34 (H1N1), was performed in a Middlebrook airborne infection apparatus (Tri-R Instruments, Rockville Centre, N.Y.). The animals were observed daily for 14 days, and the significance of the protection induced was determined by the chi-square test incorporating the Yates correction.

Preparation of spleen cell suspensions. Mice were killed by cervical dislocation, and the spleens were removed aseptically. Single-cell suspensions were made by gently teasing the spleens between two ground glass slides and pressing the cells through a 50-μm mesh. Cells were washed twice in cold Hanks balanced salt solution and suspended at a concentration of 10^7 viable cells per ml in Dulbecco minimum essential medium containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 10% fetal calf serum.

CL assay. Samples of 500 μl of spleen cell suspensions in round-bottomed vials were maintained at 37°C until used. The cell samples were mixed with 10 μl of luminol at a concentration of 1 mg/ml in phosphate-buffered saline containing 0.4% triethylamine and were incubated for 10 min at 37°C. CL measurements were performed at 37°C in the Biolumat model 9505 (Bethold, Wildbad, Federal Republic of Germany), which permits simultaneous reading of six samples. After a 3-min measurement of background, CL was generated by adding 10 μl of zymosan (Sigma, Munich, Federal Republic of Germany) suspended in buffered saline at a concentration of 50 mg/ml. CL was continuously monitored on a programmed microcomputer.

RESULTS

CL of spleen cells from mice pretreated with MDP, TDM, and the combination MDP plus TDM. Mice were pretreated by the intravenous route with the oil-in-water emulsions containing MDP, TDM, or the combination MDP plus TDM. Three weeks after pretreatment, the spleen cells were stimulated in vitro with zymosan for the measurement of CL. The results (Fig. 1) show that the CL of spleen cells from animals pretreated with 150 μg of MDP did not differ significantly from the CL from controls. In contrast, the cells from mice pretreated with 75 μg of TDM were apparently stimulated and produced more CL than the controls (Fig. 2). The synergestic combination of the two immunostimulants, 150 μg of MDP plus 75 μg of TDM, greatly enhanced the generation of CL (Fig. 3). The results at 1 and 2 weeks after pretreatment were essentially similar to those obtained at 3 weeks (data not shown).

Stimulation of nonspecific resistance against influenza virus by MDP and analogs combined with TDM. The enhanced activity of the MDP-plus-TDM combination was tested by in vivo challenge with an aerosol of mouse-pathogenic A/PR/8/34 influenza virus. Oil-in-water emulsions of MDP or its 6-*O*-acyl derivatives, alone or in combination with TDM, were administered intravenously to mice 3 weeks before lethal aerosol infection.

The results of a representative experiment (Table 1) show that all mice pretreated with MDP, irrespective of the seven

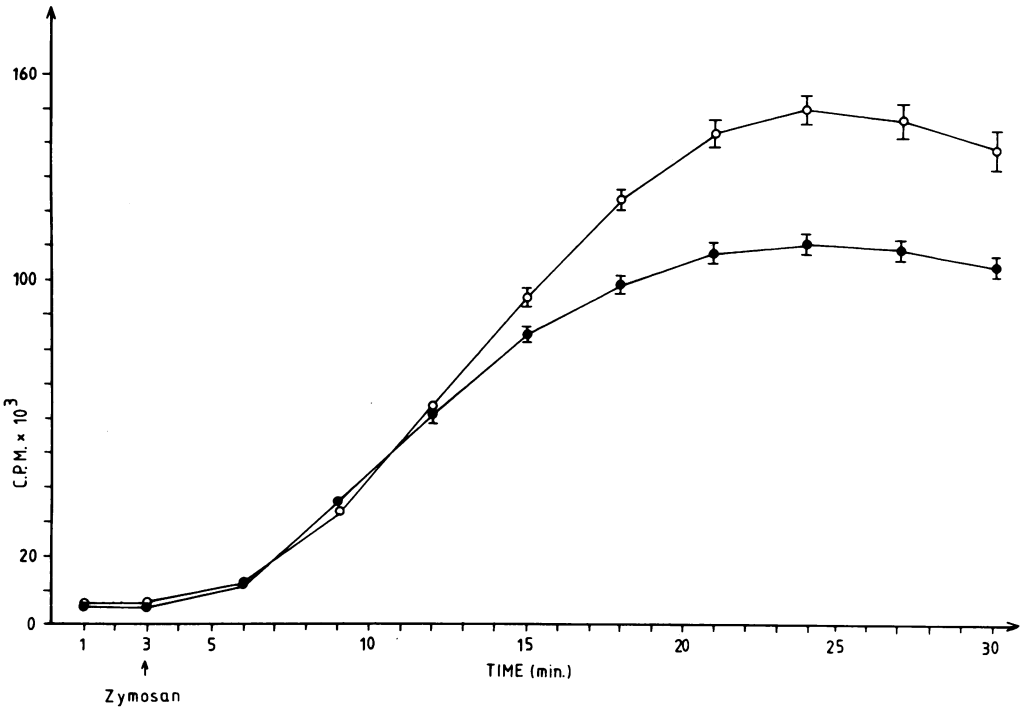

FIG. 2. Zymosan-induced CL response of spleen cells from mice pretreated with oil-in-water emulsion of TDM (○) and from control mice (●). Each point represents mean value ± standard error of three experiments.

MDP preparations used, succumbed to influenza virus. Although significant resistance was not induced by TDM alone, TDM exhibited slight activity. The combination of MDP (alanyl) with TDM conferred a significant protection against influenza virus-induced mortality. The α-branched B30-MDP conferred a high degree of resistance, in contrast to the inactive α-branched, β-hydroxylated BH48-MDP. The effect of combining MDP analogs containing differing lengths of linear fatty acid chains with TDM showed a significant protection by the longer-chain L18-MDP, whereas the short-

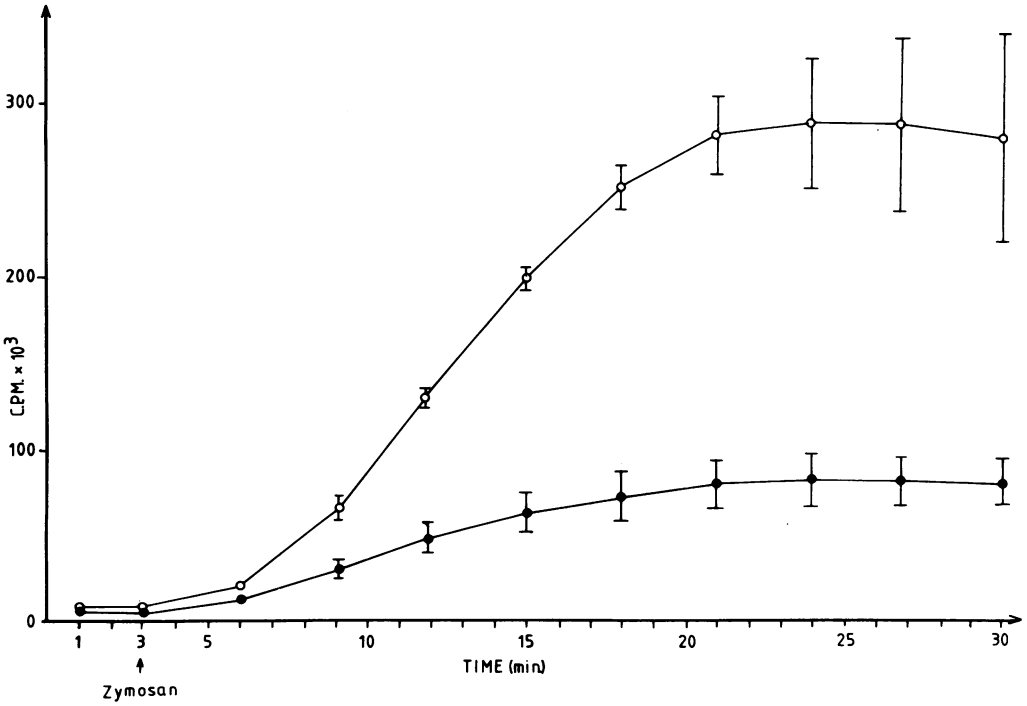

FIG. 3. Zymosan-induced CL response of spleen cells from mice pretreated with oil-in-water emulsion of MDP combined with TDM (○) and from control mice (●). Each point represents mean value ± standard error of three experiments.

TABLE 1. Protective effect of MDP and analogs alone and in combination with TDM against influenza virus infection[a]

MDP analog (300 μg)	TDM (100 μg)	Mean time of death (days ± SD)	No. of survivors/ total	P
MDP	−	6.5 ± 0.5	0/10	
	+	7.0 ± 0.8	6/10	<0.001
B30-MDP	−	7.2 ± 1.3	0/10	
	+	6.5 ± 0.7	8/10	<0.001
BH48-MDP	−	6.4 ± 0.5	0/10	
	+	6.5 ± 0.5	0/10	
L8-MDP	−	6.5 ± 0.7	0/10	
	+	6.6 ± 1.2	2/10	
L18-MDP	−	6.6 ± 0.8	0/10	
	+	7.0	8/10	<0.001
Iso.15-MDP	−	6.3 ± 0.7	0/10	
	+	6.5 ± 0.6	6/10	<0.001
QS-10-MDP-66	−	6.9 ± 0.3	0/10	
	+	7.0 ± 0.7	5/10	<0.01
Vehicle control (squalane in saline)	−	6.6 ± 0.8	0/20	
	+	7.1 ± 1.0	2/10	

[a] Mice were treated intravenously with various squalane-in-water preparations and were given 100% lethal doses of mouse-pathogenic A/PR/8/34 (H1N1) influenza virus as an aerosol 3 weeks after pretreatment.

chain analog L8-MDP induced no activity. A significant resistance to influenza virus could also be conferred by the combination with TDM of two MDP derivatives, Iso.15-MDP acylated with *Propionibacterium acnes* cell wall peptidoglycan isopentadeconic acid, and QS-10-MDP-66 containing an ubiquinone compound.

DISCUSSION

MDP and TDM can enhance the cell-mediated immune responses when administered in mineral oil emulsions (4, 8). However, the combination of MDP and TDM in an oil-in-water emulsion has proved to be particularly effective for immunopotentiation. A strong antitumor activity, with nearly 100% cures against established line 10 hepatoma tumors of strain 2 guinea pigs, has been obtained with the emulsions of MDP derivatives plus TDM, whereas preparations containing MDP only or TDM only were ineffectual (20). Similar results have been obtained in the line 10 hepatocarcinoma of strain 2 guinea pigs injected intralesionally with MDP plus TDM over a wide dose range (29).

The regression of tumors in experimental animal models has led to clinical trials of mycobacterial cell wall skeleton plus TDM for immunotherapy of malignant melanoma (27) and lung cancer (9). Host resistance against microbial infections has also been stimulated by emulsions of MDP plus TDM. The intravenous injections of MDP plus TDM preparations administered 3 weeks before aerosol influenza virus infection were shown to confer complete protection, comparable to specific immunization with an inactivated influenza virus vaccine (18). A significant protection against an aerogenic tuberculosis infection was conferred by the combination MDP plus TDM but not by either compound administered separately (3).

The results of the present study show that spleen cells from mice pretreated with TDM alone, but not those from mice pretreated with MDP alone, generated increased CL. In another study, the macrophages elicitated by TDM have been shown to release substantial hydrogen peroxide and to express antitumor activity (16). In this connection, it is of interest that the mice pretreated intravenously with 150 μg of an MDP oil-droplet preparation provided a borderline protection (50%), whereas a dose of 75 μg of similarly prepared TDM provided significant protection (75%) against an aerosol influenza virus infection which caused 80% mortality in controls (18). In the present experiments, a marginal effect was observed after a lethal (100%) aerosol influenza virus infection in animals pretreated with TDM but not in those pretreated with MDP.

The enhancement of the nonspecific host resistance to infection by MDP and its analogs is closely related to chemical structure (5, 19). In the present experiments, several 6-*O*-acyl and one ubiquinone derivative of MDP were tested for the induction of a relatively long-term nonspecific resistance against a lethal aerosol influenza virus infection. Regardless of the MDP analog used for the pretreatment, there was no evidence of any resistance against the influenza virus infection. However, in combination with TDM, MDP and several analogs conferred a significant protection against the influenza virus-induced mortality of mice. The combination of TDM with B30-MDP, containing an α-branched fatty acid chain, or L18-MDP, containing a long chain of fatty acids, induced a high degree of nonspecific resistance against the influenza virus infection. Both these lipophilic MDP derivatives have been found to confer nonspecific resistance against *Escherichia coli* infection (19), and L18-MDP has been shown to induce a protective activity against *Pseudomonas aeruginosa* pneumonia in immunosuppressed guinea pigs (22). The combination of TDM with BH48-MDP, containing an α-branched, β-hydroxylated fatty acid chain, or with L8-MDP, containing a short chain of fatty acids, did not stimulate significant resistance against influenza virus infection in the present experiment, and administration of these two MDP analogs was found to induce only a weak activity or no activity against *E. coli* infection (19).

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