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Stimulation of Chemiluminescence by Synthetic Muramyl Dipeptide and Analogs

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The effect on respiratory burst of murine splenic cells after in vitro exposure to synthetic muramyl dipeptide (MDP) and 6-*O*-acyl and quinonyl derivatives was studied at an early phase of interaction by luminol-dependent chemiluminescence (CL) in response to stimulation by zymosan. The MDP molecule enhanced CL, but the degree of CL response varied with the kinds of fatty acids introduced in the chemical structure of synthetic glycopeptide analogs. A 6-*O*-acyl derivative possessing an α-branched fatty acid chain, B30-MDP, stimulated maximum levels of CL activity. High CL responses were obtained with L8-MDP having a short chain of linear fatty acids and with QS-10-MDP-66 containing a ubiquinone compound. CL was also stimulated by MDP and its analogs in the spleen cells of nude mice lacking mature T lymphocytes, but the extent of stimulation was decreased compared with that of normal spleen cells.

Synthetic muramyl dipeptide (MDP) is the minimum adjuvant structure that can substitute for *Mycobacterium* sp. in Freund complete adjuvant (9, 14). The administration of MDP in vivo or in vitro can enhance both humoral (4, 8) and cell-mediated (5, 6, 19) immune responses.

The macrophage appears to be one of the target cells for the immunopotentiating activity of MDP. Recent studies have demonstrated that MDP can inhibit macrophage migration (21); enhance the production of T-cell-activating monokines (11, 23), collagenase and prostaglandins (34), and superoxide anion (26); and augment the cytolytic activity of macrophages (12, 31, 33).

The stimulation of oxidative metabolism in the phagocytic cell results in 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 (2, 7). The release of energy in the form of light, termed chemiluminescence (CL), can be amplified by the oxidation of cyclic hydrazide luminol by the reactive oxygen species and can be measured within a few seconds after in vitro stimulation (1). Studies with ^{14}C-labeled MDP (28) and ^{3}H-labeled MDP (3) have demonstrated the rapid elimination of 90 to 95% intact MDP in urine within 2 h. It has been suggested that the biological effect of MDP may be due to an immediate action at the cellular level (28). The present experiments were performed to study the effect on respiratory burst of MDP and its analogs at an early phase of interaction with murine splenic cells using a luminol-dependent CL assay.

MATERIALS AND METHODS

Animals. Six-week-old BALB/c mice were obtained from the specific-pathogen-free colony maintained by the Bundesgesundheitsamt. Nude mice of BALB/c background were purchased from Bomholtgard, Ry, Denmark.

Synthetic glycopeptides. Seven different compounds were used in these studies. *N*-acetyl muramyl-L-alanyl-D-isoglutamine (MDP) was purchased from Institut Pasteur Production, Paris, France. 6-*O*-(2-tetradecylhexadecanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (B30-MDP) and 6-*O*-(3-hydroxy-2-docosylhexacosanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (BH48-MDP) were synthesized by the method of Kusumoto et al. (15); 6-*O*-octanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (L8-MDP), 6-*O*-stearoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (L18-MDP), and 6-*O*-isopentadecanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (Iso.15-MDP) were synthesized by the method of Kusumoto et al. (16); and 2,3-dimethoxy-5-methyl-6-(9′-carboxynoyl)-1,4-benzoquinone-*N*-acetyl muramyl-L-valyl-D-isoglutamine-methyl ester (QS-10-MDP-66) were synthesized by the method of Kobayashi et al. (13). All preparations were weighed and dissolved or suspended in distilled water. Suspensions were dispersed by ultrasonic treatment just before use.

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 concentra-

tion of 1×10^7 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 (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 incubated for 10 min at 37°C. CL measurements were performed at 37°C in a newly developed Biolumat model 9505 (Berthold, Wildbad, Germany), which permits the simultaneous reading of six samples. After the measurement of background for 3 min, CL was generated by the addition of MDP or saline, followed immediately by 10 μl of nonopsonized zymosan (Sigma Chemical Co., München, Germany) suspended in buffered saline at a concentration of 50 mg/ml. CL was continuously monitored on a programmed microcomputer.

RESULTS

Dose-dependent stimulation of CL by MDP. Initial experiments showed that MDP itself apparently does not generate CL since the low values obtained after the direct addition of MDP alone were not significantly different from those of saline controls (unpublished data). The effect of various doses of MDP was therefore determined by the stimulation of zymosan-induced CL. The results (Fig. 1) show that a significant level of CL was stimulated by even 1 μg of MDP, although 100 μg elicited the highest CL response. A high MDP dose of 1,000 μg had an inhibitory effect on CL. Zymosan-stimulated CL counts (± standard error) without MDP were

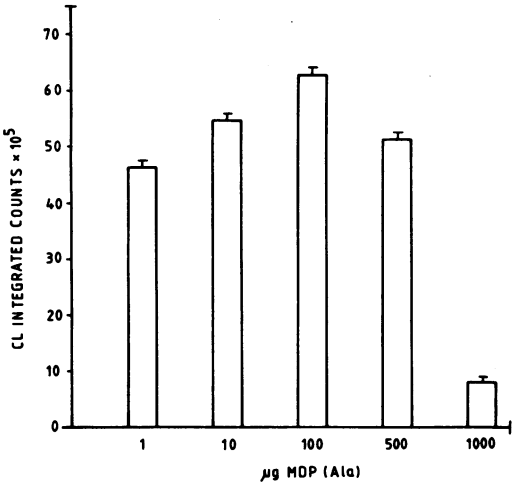

FIG. 1. Effect of concentration of MDP on stimulation of zymosan-induced CL of spleen cells. The results are the means of three experiments. The bars indicate the standard error of the mean.

$35.8 \pm 6.5 \times 10^5$. All further experiments were performed with 100 μg of MDP and an equivalent dose of analogs. Spleen cell suspensions containing MDP displayed enhanced CL response to zymosan stimulation compared with saline controls (Fig. 2).

Stimulation of CL by MDP analogs. The CL response by α-branched B30-MDP and α-branched, β-hydroxylated BH48-MDP is shown in Fig. 3. Significantly high CL activity was stimulated by B30-MDP, whereas BH48-MDP

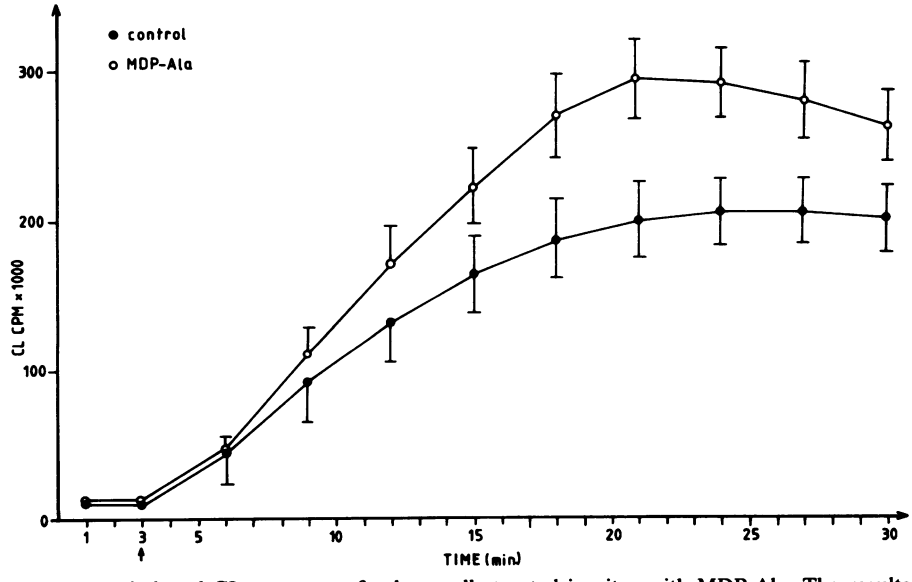

FIG. 2. Zymosan-induced CL response of spleen cells treated in vitro with MDP-Ala. The results are the means of nine experiments. The bars indicate the standard error of the mean.

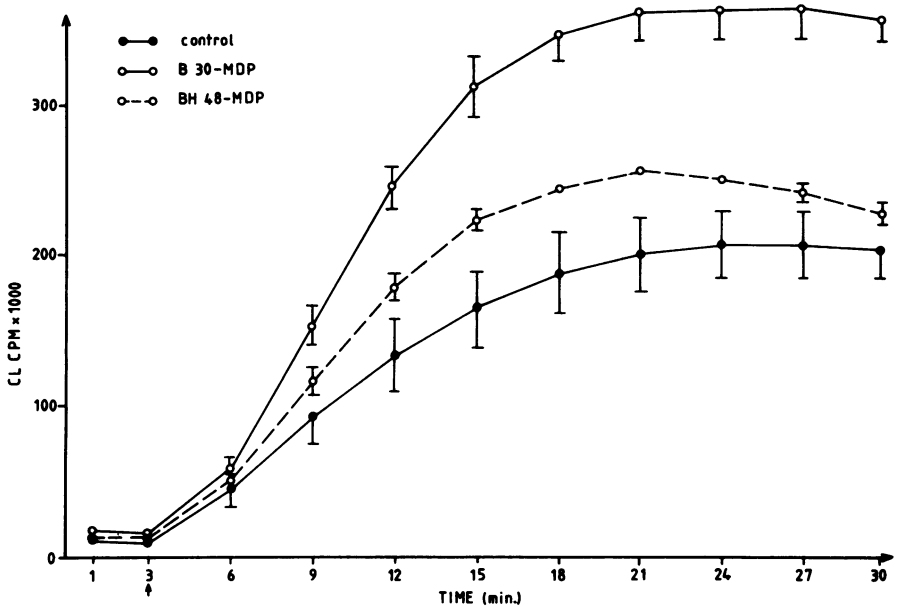

FIG. 3. Zymosan-induced CL response of spleen cells treated in vitro with B30-MDP and BH48-MDP. The results are the means of three experiments. The bars indicate the standard error of the mean.

enhanced CL only to a limited extent. The effect on CL of MDP analogs containing different lengths of linear fatty acid chains was investigated with L8-MDP and L18-MDP. The results (Fig. 4) show a higher CL stimulation by L8-MDP, possessing a shorter fatty acid chain, than by L18-MDP, possessing a longer chain. The latter preparation exhibited depressive action on CL activity when compared with controls, although the viability of spleen cells as determined by the trypan blue exclusion test was not reduced. MDP derivative Iso.15-MDP acylated with *Propionibacterium acnes* cell wall peptidoglycan isopentadecanoic acid and QS-10-MDP-

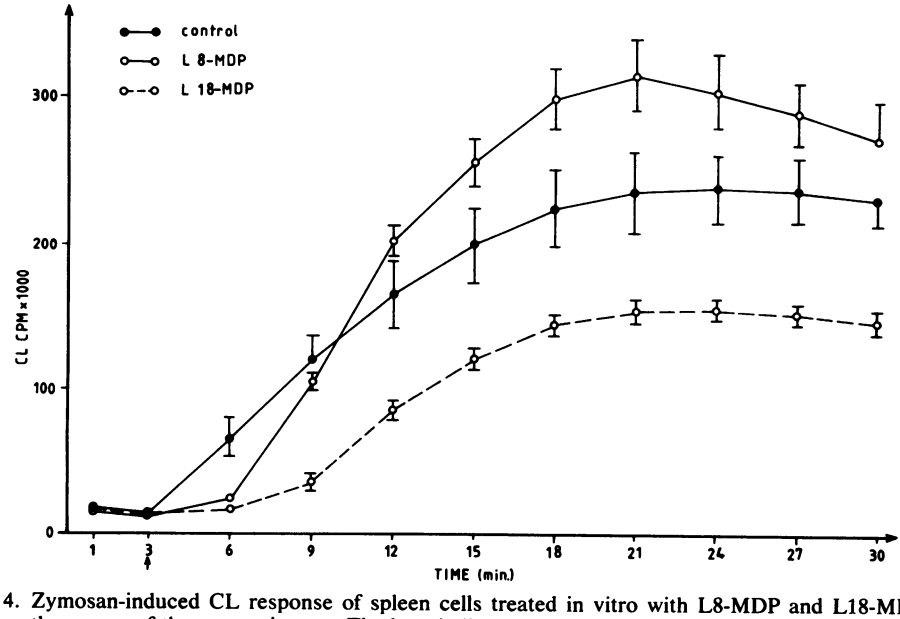

FIG. 4. Zymosan-induced CL response of spleen cells treated in vitro with L8-MDP and L18-MDP. The results are the means of three experiments. The bars indicate the standard error of the mean.

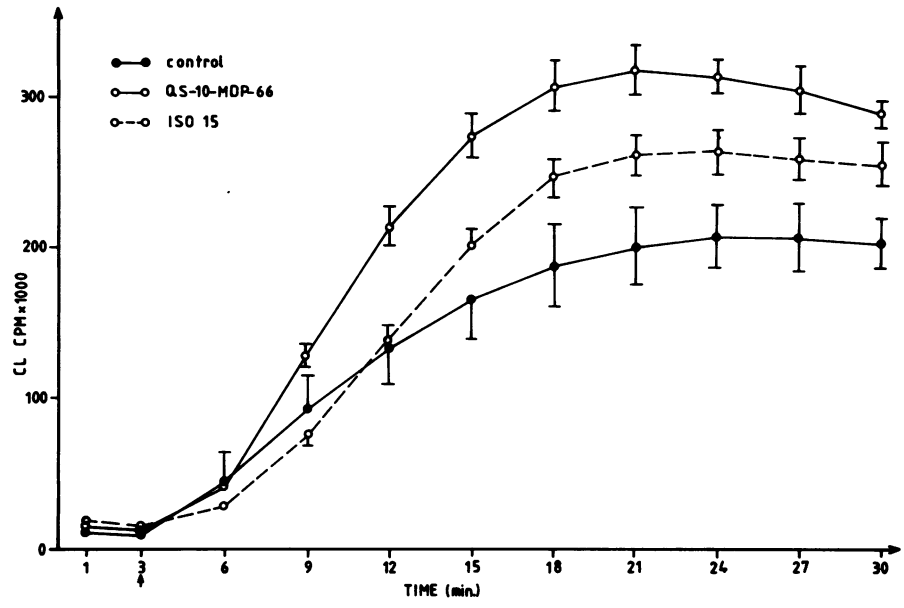

FIG. 5. Zymosan-induced CL response of spleen cells treated in vitro with QS-10-MDP-66 and Iso.15-MDP. The results are the means of three experiments. The bars indicate the standard error of the mean.

66 containing an ubiquinone compound were also tested by the CL assay. Figure 5 shows the stimulation of CL by both preparations.

Stimulation of CL by MDP and analogs in nude mice. The role of T cells in present CL experiments was evaluated by using spleen cell suspensions from nude mice lacking mature T lymphocytes. The stimulation of CL in nude cells by MDP, L8-MDP, and QS-10-MDP-66 in comparison with normal BALB/c spleen cells is shown in Fig. 6. Nude spleen cells gave higher CL counts than did normal cells with the three MDP derivatives tested. However, the increase in the CL stimulated by MDP and its analogs in relation to their respective controls was clearly reduced in the nude cells as compared with normal spleen cells.

DISCUSSION

The present study demonstrates that the zymosan-induced CL response of murine splenic cells can be enhanced by MDP within 30 min. Experiments with ^{14}C-labeled MDP have shown that after intravenous injection in mice, a rapid clearance of 50 to 65% radioactivity occurs within 30 min and 75 to 95% occurs by 2 h (28). After the injection of tritium-labeled MDP, 95% of MDP, found to be intact on high-pressure liquid chromatography, was recovered in urine also within 2 h (3). The present investigation of the early phase of interaction between MDP and murine splenic cells lends support to the suggestion that the biological effects of MDP may result from an immediate and sustained action at the cellular level (28).

Of the seven different MDP preparations tested, B30-MDP containing an α-branched fatty acid chain showed maximum stimulation of CL. B30-MDP has also been found to be more active than MDP in stimulating macrophages to release superoxide anion in a cytochrome *c* assay (25). In contrast, BH48-MDP containing α-branched, β-hydroxylated fatty acids stimulated CL only to a limited extent. Interestingly, the intraperitoneal administration of B30-MDP induced nonspecific resistance against *Escherichia coli* infection in mice, but BH48-MDP was without effect (18).

L8-MDP, possessing a shorter fatty acid chain, stimulated CL, whereas longer-chain L18-MDP had an inhibitory effect on CL. Both derivatives, however, conferred nonspecific resistance against *E. coli* infection (18, 24). This discrepancy may be explained, in part, by the detergent effect of L18-MDP, which is lacking in L8-MDP. The acylation of L18-MDP to increase lipophilicity causes physicochemical changes in the parent MDP molecule and detergent effects as determined by the hemolysis produced (18).

Iso.15-MDP showed limited stimulation of CL, in contrast to QS-10-MDP-66, which stimulated considerable CL activity. QS-10-MDP-66 containing a quinonyl group has been shown to be an active adjuvant for the induction of allogeneic killer T cells in mice and has tumor-suppressive activity (29).

The role of mature T lymphocytes in the

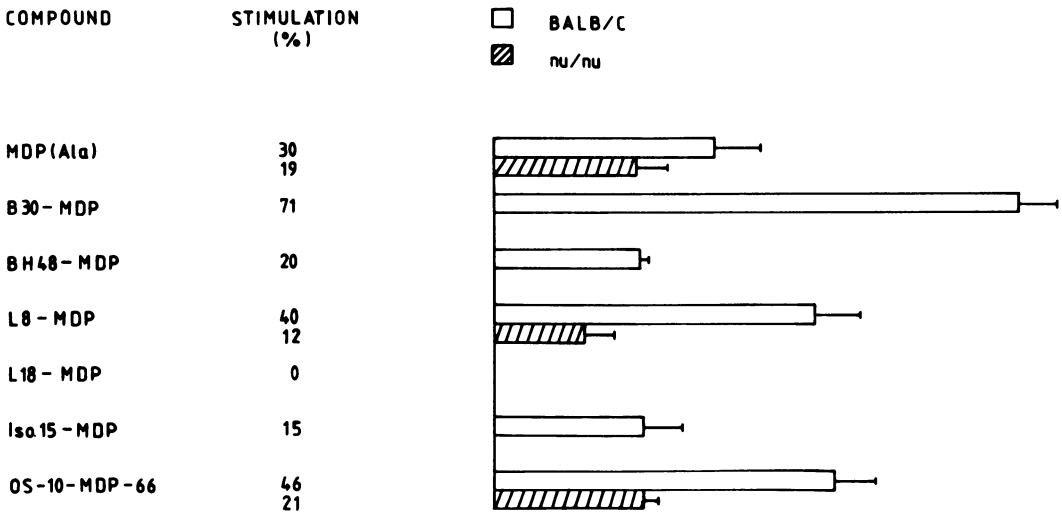

FIG. 6. Stimulation of zymosan-induced CL in spleen cells of normal and nude mice by MDP and its analogs. The results are the means of three experiments expressed as the percent increase over 100% control values. The bars indicate the standard error of the mean.

stimulation of CL was investigated in experiments with spleen cells from nude mice. CL was stimulated by MDP and its analogs in nude splenic cells, suggesting that the presence of mature T cells is not absolutely essential. MDP has been shown to activate macrophages from nude mice (26) and confer resistance against infection in neonates (27). However, mature T lymphocytes, as present in normal spleen cell suspensions, apparently augment the extent of the CL response. MDP has been shown to interact with T and B lymphocytes (17, 32). The optimal stimulation of CL by MDP seems to require interaction between macrophages and T lymphocytes.

The reduction of oxygen to superoxide anion and hydrogen peroxide by activated macrophages is associated with microbicidal and even tumoricidal activities (20, 22). Macrophage activation can be indicated by enhanced CL (30), and a correlation between microbicidal activity and CL has been reported (10). Our studies suggest that the enhanced production of toxic oxygen metabolites by MDP and its analogs, leading to the emission of light measured by zymosan CL, can be used as one of the early indicators of the stimulatory capability of MDP.

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