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N-Acetyl Muramyl Dipeptide Stimulation of Bone Resorption in Tissue Culture

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N-Acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), a structurally defined fragment of bacterial peptidoglycan, stimulated significant release of previously incorporated ^{45}Ca from fetal rat bones in tissue culture over the concentration range of 0.1 to 10.0 μg/ml. MDP-Stimulated bone resorption was not inhibited by the addition of the prostaglandin synthetase inhibitor indomethacin to the culture medium. MDP was neither mitogenic for nor stimulated the release of osteoclast-activating factor from cultured human peripheral blood mononuclear cells. Thus, MDP-stimulated bone resorption in vitro is mediated by a mechanism which is not dependent upon prostaglandins or osteoclast-activating factor. 6-O-Stearoyl-*N*-acetyl-muramyl-L-alanyl-D-isoglutamine, a lipophilic analog of MDP, was slightly more potent than MDP. Two diastereomers of MDP, *N*-acetyl-muramyl-L-alanyl-L-isoglutamine and *N*-acetyl-muramyl-D-alanyl-D-isoglutamine, which are inactive as adjuvants, were at least 1,000 times less active than MDP in stimulating bone resorption. The stereochemical specificity for bone-resorptive activity paralleled that required for adjuvant activity, macrophage activation, and activation of the reticuloendothelial system.

Bone resorption is seen adjacent to sites of bacterial infection in periodontal disease and osteomyelitis. Although bacteria are acknowledged as the etiological agent of periodontal disease (22), it is not known whether the bone resorption seen is mediated by a direct effect of bacterial products on bone tissue or whether bacterial products act indirectly through stimulation of host tissues to produce a bone-resorbing mediator such as prostaglandin E (7) or osteoclast-activating factor (OAF) (13). Because of the difficulty of examining bone resorption in vivo, resorptive mediators have been examined in vitro with tissue culture systems (19). With these in vitro methods, various bacterial cell wall components have been shown to stimulate bone resorption: lipopolysaccharide (10), lipoteichoic acid (8), and peptidoglycan (17). Each of these cell wall components is a heterogeneous, high-molecular-weight polymer. A chemically defined minimal fragment of these compounds which retains bone-resorptive activity has not been determined. The identification of a chemically defined bacterial mediator would facilitate future in vivo studies to determine the relative importance of direct and indirect mechanisms of bacterium-induced bone resorption. The minimal fragment of bacterial peptidoglycan which retains adjuvant and certain other biological activities is *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) (5, 16). If the stereochemical requirements for bone-resorptive activity of peptidoglycan fragments parallels that for other biological activities, then MDP could be the chemically defined bone-resorptive agent sought.

The purpose of the present study was to examine the ability of MDP to stimulate bone resorption in tissue culture. The results provide evidence that MDP retains the bone-resorptive activity of peptidoglycan. The stereochemical specificity required for bone-resorptive activity parallels that required for other biological effects. MDP-stimulated resorption is not dependent upon indirect mediation by prostaglandins or OAF.

MATERIALS AND METHODS

MDP and analogs. MDP and analogs were purchased from Calbiochem-Behring Corp., La Jolla, Calif.

Bone tissue culture. The fetal rat long bone tissue culture methods used have been described previously (19). Fetal bones were labeled with ^{45}Ca by injecting the mother with 200 μCi of ^{45}Ca (New England Nuclear Corp., Boston, Mass.) on day 18 of gestation. Paired radii and paired ulnae bone shafts were obtained from 19-day-old fetuses by microdissection. The paired shafts were first cultured in BGJ_b medium (GIBCO Laboratories, Grand Island, N.Y.) containing 1.0 mg of bovine serum albumin (Pentex bovine serum albumin fraction V; Miles Laboratories, Inc., Elkhart, Ind.) per ml for 1 day to reduce exchangeable ^{45}Ca. The paired shafts were then transferred into medium with and without the test agent for 5 days of culture, with one medium change at 2 days. The percentage of ^{45}Ca released during the 5-day culture was determined

by measuring the radioactivity in the two media and the trichloroacetic acid-solubilized bone with a liquid scintillation counter. Stimulated bone resorption was expressed as the difference in the percentage of ^{45}Ca released by paired treatment and control bones during the 5-day culture. The men release of ^{45}Ca from control bones was 27.9 ± 0.6 (percent ± standard error).

OAF. The stimulation of human peripheral blood mononuclear (PBM) cells to produce OAF has been described previously (12). Venous blood was obtained from healthy co-workers who had not taken aspirin or any other drug during the previous week. The blood was drawn into syringes containing 15 U of heparin (Abbott Hospitals, Inc., North Chicago, Ill.) per ml and diluted 1:1 with Hanks balanced salt solution. PBM cells were separated by density gradient centrifugation on a Ficoll-Hypaque gradient (P = 1.079; Pharmacia Fine Chemicals, Piscataway, N.J.) (2). Cells were washed three times in Eagle minimum essential medium containing 5% newborn calf serum and adjusted to a final concentration of 3×10^6 cells per ml in a culture medium consisting of RPMI 1640, 0.5% heat-inactivated, pooled human AB serum, 50 μg of gentamycin per ml, 2 mM L-glutamine, and 12.5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer at pH 7.4. Test cultures contained either concanavalin A (5 μg/ml; Sigma Chemical Co., St. Louis, Mo.) or MDP (25 μg/ml). After 72 h in culture, the cells were removed by centrifugation, and the PBM-conditioned medium was decanted. The conditioned medium was then concentrated 10 times on a 10,000-dalton cutoff ultrafiltration membrane (PM-10; Amicon Corp., Lexington, Mass.), diafiltered with 10 volumes of BGJ_b medium without albumin, and frozen at −20°C. The diafiltration step removed 492-dalton MDP, which would otherwise interfere with the resorption assay for OAF. The 10-times concentrated, PBM-conditioned medium was then serially diluted with BGJ_b medium and assayed for OAF activity by bone tissue culture assay. Culture medium and medium components were obtained from Microbiological Associates, Bethesda, Md.

Mitogenesis. PBM cell cultures were examined for mitogenic responses by measuring the incorporation of [^{3}H]thymidine (0.5 μCi per culture; specific activity, 1.9 μCi/μmol; New England Nuclear Corp.) during a 6-h pulse after a 72-h incubation with mitogen. Cells were collected on glass microfiber filters, and radioactivity was determined with a liquid scintillation counter.

RESULTS

The response of fetal rat long bones in organ culture to stimulation with MDP is shown in Table 1. MDP stimulated significant bone resorption at 0.1, 1.0, and 10 μg/ml, but not at 0.01 or 100 μg/ml.

To determine whether the structure-activity relationship for the stimulation of bone resorption was similar to that observed for immunoadjuvant activity (1, 3), macrophage activation (18, 27), and activation of the reticuloendothelial system (24), three MDP analogs were examined (Table 2). 6-O-Stearoyl-N-acetyl-muramyl-L-alanyl-D-isoglutamine, a lipophilic analog of MDP, was approximately 10 times more potent than MDP and had a broader dose-response range, stimulating bone resorption at 0.01 to 10 μg/ml. Two diastereomers of MDP, N-acetyl-muramyl-D-alanyl-D-isoglutamine and N-acetyl-muramyl-L-alanyl-L-isoglutamine, stimulated significant bone resorption only at 100 μg/ml and were thus 1,000 times less active than MDP.

Several agents that stimulate bone resorption

TABLE 1. Effect of MDP on the release of ^{45}Ca from fetal rat long bones in organ culture

Dose (μg/ml)	Pairs (n)[a]	Stimulated release[b]	
		% ± SE	P[c]
100	5	0.9 ± 5.2	0.87
10	36	23.5 ± 3.0	<0.001
1	29	24.8 ± 3.0	<0.001
0.1	17	16.6 ± 3.1	<0.001
0.03	5	12.9 ± 5.9	0.12
0.01	9	1.3 ± 3.2	0.70

[a] Number of bone pairs.
[b] Mean difference in percentage of ^{45}Ca released by paired treatment and control bones.
[c] Significance of stimulated release differing from zero with a two-tailed t test.

TABLE 2. Effect of MDP analogs on the release of ^{45}Ca from fetal rat long bones in organ culture

Dose (μg/ml)	Pairs[a]	Stimulated release[b]	
		% ± SE	P[c]
6-S-MDP[d]			
100	6	6.7 ± 3.3	0.11
10	6	29.5 ± 6.3	0.009
1	12	19.8 ± 5.2	0.004
0.1	11	22.3 ± 5.0	0.002
0.01	17	16.7 ± 3.0	<0.001
0.001	6	−5.8 ± 2.8	0.11
DD-MDP[e]			
100	11	13.8 ± 3.8	0.006
10	12	3.0 ± 1.6	0.09
1	12	1.7 ± 1.5	0.29
LL-MDP[f]			
100	12	9.1 ± 3.4	0.02
10	12	3.8 ± 2.6	0.17
1	11	1.5 ± 1.7	0.42

[a] Number of bone pairs.
[b] Mean difference in percentage of ^{45}Ca released by paired treatment and control bones.
[c] Significance of stimulated release differing from zero with a two-tailed t test.
[d] 6-S-MDP, 6-O-Stearoyl-N-acetyl-muramyl-L-alanyl-D-isoglutamine.
[e] DD-MDP, N-Acetyl-muramyl-D-alanyl-D-isoglutamine.
[f] LL-MDP, N-Acetyl-muramyl-L-alanyl-L-isoglutamine.

TABLE 3. Effect of indomethacin on MDP-stimulated release of ^{45}Ca from fetal rat long bones in organ culture

Indomethacin (μg/ml)	Pairs[a]	Stimulated release[b]	
		% ± SE	P[c]
MDP (1 μg/ml)			
0	29	24.8 ± 3.0	<0.001
0.1	6	19.1 ± 6.6[c]	0.004
1	16	21.4 ± 3.6[c]	<0.001
10	18	26.8 ± 2.9[c]	<0.001

[a] Number of bone pairs.
[b] Mean difference in percentage of ^{45}Ca released by paired treatment and control bones.
[c] Significance of stimulated release differing from zero with a two-tailed *t* test.
[d] Not significantly different from the stimulated release of the group with zero indomethacin.

do so through prostaglandin-mediated mechanisms: phorbol esters (25), melettin (25), complement (20), and arachidonic acid (6). The resorption stimulated by each of these agents is blocked by the addition of the prostaglandin synthetase inhibitor indomethacin to the culture system. Because MDP is known to stimulate prostaglandin production by macrophages (27), it seemed possible that MDP-stimulated bone resorption could be mediated indirectly through a prostaglandin-dependent mechanism. I therefore examined the ability of indomethacin to affect MDP-stimulated bone resorption. Resorption stimulated by 1 μg of MDP per ml (Table 3) was not inhibited by the addition of 10^{-7}, 10^{-6}, or 10^{-5} M indomethacin to the culture medium.

Because of reports that MDP is mitogenic for lymphocytes under certain conditions (23) and because lipopolysaccharide and other mitogens can stimulate lymphocytes to produce the bone-resorbing lymphokine OAF, (13, 26), I examined the ability of MDP to stimulate OAF production and mitogenesis in PBM cells. MDP at 25 μg/ml did not stimulate the release of OAF into the culture media of PBM cells (Table 4). When MDP was added at the end of the culture period, no resorptive activity was seen. This shows that MDP was removed by diafiltration and that without stimulation, PBM cells do not release OAF activity. The positive control, concanavalin A at 5 μg/ml, stimulated PBM cells to release significant OAF activity into the culture medium. The bone resorption produced by the addition of concanavalin A-stimulated, PBM-conditioned medium was highly significant at dilutions of 1:1.5 and 1:4.5.

The proliferative response of PBM to MDP and concanavalin A is shown in Table 5. MDP at 25 μg/ml was not significantly mitogenic, having a stimulation index of only 1.4. The positive control, concanavalin A, was highly mitogenic, with a stimulation index of 75.

DISCUSSION

These studies show that MDP, a small chemically defined fragment of peptidoglycan common to most bacteria (21), stimulates bone resorption in tissue culture. This finding, plus the finding that whole group A streptococcal peptidoglycan stimulates bone resorption (17), raises the possibility that intermediate-sized fragments of peptidoglycan and peptidoglycans from various bacterial sources may also have bone-resorptive activity. If linkage between adjuvant activity and bone-resorptive activity is substantiated by further research, then the reports that the peptidoglycan of several gram-positive (14)

TABLE 4. Effect of PBM cell-conditioned media on ^{45}Ca release from fetal rat long bones

PBM culture treatment	Dilution (ml/ml)[a]	Pairs[b]	Stimulated release[c]	
			% ± SE	P[d]
MDP (25 μg/ml) added at end of PBM culture	1:1.5	6	−0.8 ± 0.5	0.20
	1:4.5	4	2.5 ± 2.5	0.39
MDP (25 μg/ml) added at start of PBM culture	1:1.5	10	4.4 ± 3.1	0.19
	1:4.5	12	0.7 ± 1.8	0.70
	1:13.5	6	2.3 ± 2.0	0.31
Concanavalin A (5 μg/ml) added at start of PBM culture	1:1.5	12	36.8 ± 5.1	<0.001
	1:4.5	12	24.9 ± 4.8	<0.001
	1:13.5	6	10.8 ± 6.2	0.14

[a] Final effective dilution of PBM media in bone tissue culture media.
[b] Number of bone pairs.
[c] Mean difference in percentage of ^{45}Ca released by paired treatment and control bones.
[d] Significance of stimulated release differing from zero with a two-tailed *t* test.

TABLE 5. Effect of MDP on PBM cell proliferation

Mitogen (μg/ml)	Stimulation index		
	n^a	cpm ± SE	P^b
None	6	1.0 ± 0.17	
MDP (25)	6	1.4 ± 0.11	0.09
Concanavalin A (5)	6	75.2 ± 1.67	<0.001

[a] *n*, Number of cultures.
[b] Significance of difference from no mitogen by two-tailed *t* test.

and gram-negative bacteria (4) possess adjuvant activity would have direct implications for bacterium-induced bone resorption. Peptidoglycan fragments may be responsible for the bone-resorbing activity of *Actinomyces viscosus* (9) and of a partially characterized bone-resorbing mediator obtained from inflamed dog gingiva (11). However, just as it has been observed that peptidoglycans from some bacteria do not possess adjuvant activity (14), I have observed that cell wall materials from some bacteria do not have bone-resorptive activity (unpublished data). The specific structure of a whole peptidoglycan and the structure of the fragments produced by digestion with bacterial and mammalian enzymes have been shown to markedly affect adjuvant activity (15) and may similarly be expected to affect bone-resorptive activity.

The stereochemical specificity required for adjuvant activity (28), macrophage activation (27), and activation of the reticuloendothelial system (24) is also required for bone-resorptive activity (Table 2). The lack of bone-resorbing activity of *N*-acetyl-muramyl-D-alanyl-D-isoglutamine and *N*-acetyl-muramyl-L-alanyl-L-isoglutamine except at 100 μg/ml correlates with the lack of activity of these diastereomers in the other biological systems. The weak activity of these two diastereomers could be due to either slight intrinsic activity or to a likely contamination with as little as 0.1% of MDP. The greater potency of the lipophilic analog 6-O-stearoyl-*N*-acetyl-muramyl-L-alanyl-D-isoglutamine than that of MDP has previously been reported for the stimulation of antibody production in mice (24).

The lack of an effect of indomethacin on MDP-stimulated bone resorption (Table 3) indicates that in vitro MDP-stimulated resorption is not dependent on an indirect mechanism mediated by prostaglandins. However, since it has been demonstrated that MDP can stimulate prostaglandin and collagenase release from macrophages (27), it is possible that in vivo, a component of MDP-stimulated resorption is mediated indirectly by prostaglandins and other factors released from macrophages.

The inability of MDP to stimulate PBM cells to release OAF activity into the culture medium (Table 4) indicates that MDP-induced bone resorption is unlikely to be mediated indirectly through an OAF-dependent mechanism.

Although MDP has been reported to be mitogenic for mouse splenocytes (23), the results presented here (Table 5) are similar to those of others who have found MDP to be essentially without mitogenic activity (28).

As a structurally defined molecule, MDP should prove useful in determining the role of bacterial products in periodontal disease and other conditions in which bacteria are found in sites adjacent to bone resorption. Previous reports on the activity of MDP have generally come from the field of immunology and have examined the effect of MDP on B-cells, T-cells, macrophages, and cells of the reticuloendothelial system. It is hoped that this report on the effect of MDP on bone resorption will expand the scope of MDP research.

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