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Stimulatory Effect of *N*-Acetyl Muramyl Dipeptide In Vivo: Proliferation of Bone Marrow Progenitor Cells in Mice

BERNARD WUEST AND ERNST D. WACHSMUTH*

Research Department, Pharmaceuticals Division, CIBA-GEIGY Ltd., 4002 Basel, Switzerland

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The effects of single and multiple injections of *N*-acetyl muramyl dipeptide (MDP) on peripheral leukocytes, colony-forming cells (i.e., bone marrow granulocyte-macrophage progenitor cells), and the humoral immune response (to bovine serum albumin) were investigated in mice. Whereas low doses of MDP (0.1 to 1 mg/kg) provoked lymphocytosis, larger doses (10 mg/kg upward) resulted in lymphocytopenia and an increase in the number of young stab neutrophils and monocytes. MDP induced a dose-dependent increase in the number of bone-marrow macrophage progenitor cells, the maximum being reached at a dose around 10 mg/kg. A 50% increase in the maximum effect was produced by a dose around 0.1 mg/kg. The higher the dose, the longer the increase in these progenitor cells persisted. MDP mediated a dose-dependent antibody response to small amounts of bovine serum albumin, correlating with the proliferation of progenitor cells.

N-Acetyl muramyl dipeptide (MDP) is a component of the bacterial cell wall, which has recently been synthesized (13). It has been investigated intensively because of its ability to substitute in immunomodulation for *Mycobacterium tuberculosis* emulsified in complete Freud adjuvant (3, 13). This small natural cell wall unit and a large number of chemical analogs have subsequently been shown to be beneficial to the recipient, in that they increase host resistance, e.g., they enhance the humoral immune response, delayed-type hypersensitivity, resistance to infections, and tumor rejection (5). In addition, MDP has been found to be tolerated better than lipopolysaccharides (LPS) or lipid A, two other well-known immunomodulators (15).

The mechanism of the action of MDP is poorly understood. The cells on which it acts have not been unequivocally identified. It has been reported that MDP stimulates the reticuloendothelial system (22, 28) and inhibits macrophage migration (14), directly augments the cytolytic activity of macrophage cell lines (23), and activates macrophages (27). Moreover, MDP reputedly induces T-helper cells (11) and might interact initially either with T-lymphocytes or with B-lymphocytes (or with both) without participation of macrophages (21). On the other hand, MDP is known to be pyrogenic (19), to provoke a transient leukopenia (8), and to induce the production of a granulocyte-macrophage colony-stimulating activity (CSA) in macrophage cultures (20). The question arises whether MDP stimulates the proliferation of progenitor cells in the bone marrow, leading to an increase of mononuclear cell counts and thereby to an increased efficiency of the reticuloendothelial system.

In an attempt to answer this question, the changes in the number of peripheral leukocytes, bone marrow cells, and colony-forming units in culture (CFU-C), i.e., granulocyte-macrophage progenitor cells, in bone marrow were investigated in mice given single or multiple injections of MDP.

MATERIALS AND METHODS

Chemicals. Dulbecco minimal essential medium (10× concentrated, no. 330–205) was obtained from Gibco-Biocult Ltd. (Paisley, Scotland); methyl-cellulose (methocel MC 4000 cp) was obtained from Fluka (Buchs, Switzerland); and horse serum was obtained from Microbiological Associates (Bethesda, Md.). Penicillin and streptomycin were purchased from Calbiochem (La Jolla, Calif.), and TC-glutamine and lipopolysaccharide W from *Salmonella typhosa* were obtained from Difco Laboratories (Detroit, Mich.). MDP (*N*-acetyl-muramyl-L-alanyl-D-isoglutamine) as described by Merser et al. (13) and the MDP analog 2-acetamido-2-deoxy-3-0-[(L-1-(D-1,3-bis-methylcarbamoyl-propyl) - carbamoylethyl) - carbamoylmethyl]-D-glucose were synthesized by A. Hartmann and J. Stanek (CIBA-GEIGY Ltd, Basel, Switzerland).

MDP was dissolved in sterile water (125 mg/ml), neutralized with 1 N NaOH, filtered through a 20-μm Nalgene filter (Sibron/Nalge, Rochester, N.Y., USA), and diluted with sterile 0.9% saline (Vifor SA, Geneva, Switzerland) as required. Bovine serum albumin (BSA) was purchased from Behringwerke AG, Marburg, Germany.

Culture medium. The standard culture medium consisted of Dulbecco minimal essential medium supplemented with amino acids, vitamins, and antibiotics

(15). For bone marrow cell cultures, the standard medium contained 0.8% methocel, 15% horse serum, and 5 to 10% mouse lung-conditioned medium (MLCM) as source of CSA (23).

Animals and injection schedules. BALB/c (specific-pathogen-free) mice (Bomholtgård, Denmark), weighing 25 to 30 g, were kept in laminar flow units and received water and food (Nafag, St. Gallen, Switzerland) ad libitum. They were injected subcutaneously (0.2 or 0.5 ml) in the nape between 8 a.m. and 9 a.m. unless otherwise stated. Each group at any time or dose or both comprised 5 to 10 MDP-treated mice and 3 to 5 controls injected with saline. To obtain normal values, five mice kept under identical conditions but not injected with any compound were autopsied with the treated mice. All mice in each group were investigated simultaneously to minimize diurnal or day-to-day variations.

Sampling of cells. Mice were anesthetized with ether, and one eye was removed, and the blood was collected in heparinized tubes. They were then decapitated, and peritoneal cells were obtained as washouts of the peritoneal cavity (25). Spleen cell suspensions were prepared by teasing spleens in standard culture medium, and bone marrow cells were flushed out with 2 ml of medium from femurs previously immersed in disinfectant. After centrifugation (800 × *g*, 4°C, 7 min), the cells were suspended in culture medium to give suspensions containing 5×10^6 cells per ml.

Cell counts and cell volumes. Cell counts and cell size distributions were determined with a Coulter Counter model Z_f (Coulter Electronics Ltd., Harpenden, England) coupled to a Coulter C-1000 channelyzer and a Coulter X-Y recorder II calibrated with latex particles. Leukocyte differentials were determined in blood smears after automatized Pappenheim staining and counting 100 cells. Normalized leukocyte counts of treated mice are given as a percentage of their controls, i.e., mice injected at the same time with saline, and neutrophil and lymphocyte counts as portions of this percentage, to eliminate day-to-day and diurnal variations and the effect of the solvent saline (Fig. 1).

CFU-C determination. Bone marrow cells (BMC) (50,000 cells per ml) were cultured in 3.5-cm-diameter Greiner (Nürtingen, Germany) culture dishes (24). Spleen cells and peritoneal cells were cultured under the same conditions at a density of 10^6 cells per ml and dish. Colonies consisting of 50 or more cells were counted microscopically at a 40× magnification 7 days after setting up of the culture (37°C; 10% CO_2). Cultivation of BMC from nontreated mice, using four batches of MLCM at concentrations of 5 to 10%, led to 40 to 60% colonies with macrophages, 10 to 30% with granulocytes, and 20 to 50% with both cells.

Morphology of cells in colonies. The morphology of cells in colonies was determined after the cells had been stained in situ for aminopeptidase activity. One volume of buffered substrate-coupler solution (25) was mixed with one volume of 1% agarose, and 2 ml of this mixture was layered over the culture broth in the dishes. The enzymatic reaction was stopped, and the cells were fixed with 3% formaldehyde in phosphate-buffered saline for 1 h. Fixed stained colonies were then scored with a Leitz stereomicroscope. With conventional microscopy, at 40× magnification, colonies with only macrophages (all cells aminopeptidase positive) could be discriminated from mixed colonies (containing also aminopeptidase-negative cells) and granulocyte colonies (all cells aminopeptidase negative, most cells with a polymorphous nucleus as shown by subsequent microscopy of individual colonies).

CSA determination in serum. Serum (0.2 ml) was mixed with chloroform (0.8 ml), left for 1 h, centrifuged (5 min at 9,000 × *g*), and the chloroform was removed under sterile conditions. The serum was then again centrifuged (50 min at 9,000 × *g* at 4°C), and the extracted serum (175 μl) was left over night in a wet chamber. Fifty microliters of this extracted serum was mixed with 0.5 ml standard medium containing 0.3% agarose (25). Target bone marrow cells were cultured as described above, but in the presence of 20% horse serum, on the gelled and 3-h-incubated agarose layer, and colonies were counted after 7 days of incubation (37°C; 10% CO_2). Positive controls consisted of mice

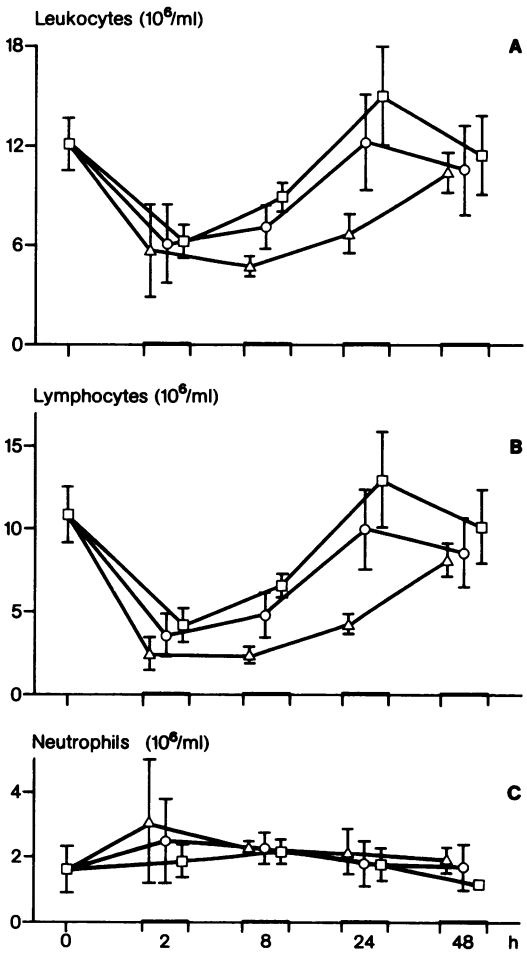

FIG. 1. Blood leukocytes after one subcutaneous MDP injection. Mice were injected at different times of the day, and all were sacrificed at 1 p.m. Means and standard deviations (vertical bars) for five mice after injection of saline (□), 1 mg of MDP per kg (○), and 100 mg of MDP per kg (△). Abscissa: hours after injection; ordinate: cell count (10^6/ml of blood).

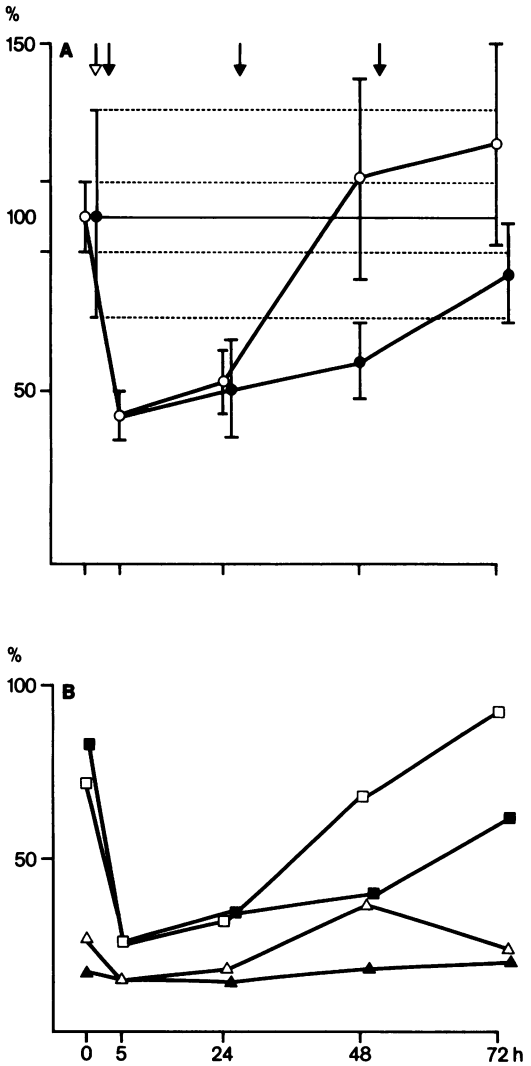

FIG. 2. Blood leukocytes after one and three MDP injections. One single injection of 1,000 mg of MDP per kg (open symbols: blood leukocytes 7.7 ± 0.8 × 10^6/ml at 0 h) and once daily for 3 days (closed symbols: blood leukocytes 5.6 ± 1.7 × 10^6/ml at 0 h). The arrows indicate the time of injection. Means and standard deviations (vertical bars) for five mice are given. Abscissa: hours after the first injection; ordinate: cell counts in percentage of leukocyte counts of corresponding saline controls (A) and the neutrophil (triangle) and lymphocyte (square) portions of the normalized leukocyte counts (B).

postendotoxin serum and normal serum mixed with 50 μl of MLCM.

Limulus amoebocyte endotoxin test. Possible contamination of MDP by LPS was checked with the standardized *Limulus amoebocyte* lysate test from Mallinckrodt (St. Louis, Mo.), using dilutions of a standard *Escherichia coli* LPS (Mallinckrodt) to obtain a quantitative evaluation. Results were judged after 15, 30, and 60 min of reaction.

Anti-BSA antibody determination in serum. Mice were immunized with a low dose of BSA (10 μg/mouse intraperitoneally) with or without MDP treatment (subcutaneous), and blood was collected 27 days thereafter. Anti-BSA antibody titers were determined in individual mouse sera by passive hemagglutination of freshly prepared glutaraldehyde-coupled BSA-sheep erythrocytes in microtiter plates (1). Titers are expressed as $\log_2$ of the reciprocal of the final agglutination dilution. No antibody was detected in sera of mice which received only BSA.

Evaluation of data. The difference between a plateau response and no effect was graphically determined from MDP dose-response curves (log MDP dose versus measure of response). The dose causing 50% of the maximal effect was then read from the graph and defined as the 50% effective dose. Means and standard deviations, but not standard error of the mean, were calculated. The student *t*-test was used for the comparison of two means.

RESULTS

Peripheral leukocytes after MDP injection. The blood leukocyte counts of mice injected with MDP and with saline were the same within the first 2 h after injection (Fig. 1A). Although leukocyte counts dropped in the given example, at other times of the year they increased. In both cases a relative leukopenia became obvious only 8 h after the injection of MDP. The relative leukopenia was apparently dose dependent and due to low lymphocyte counts (Fig. 1B) without measurable effect on neutrophil counts (Fig. 1C). The leukocyte counts returned to normal values within 48 h. In view of the response of mice injected with saline alone, the results from MDP-injected animals were, therefore, expressed as percentages of the saline controls investigated at the same time.

Leukocyte counts after one injection of 1,000 mg of MDP per kg returned to normal within 2 days (Fig. 2A). However, daily injections repeated at 24-h intervals delayed normalization (Fig. 2A). The changes in leukocyte counts reflected the dramatic drop in blood lymphocytes after the injection. Lymphopenia persisted for at least 24 h and also after repeated injections (Fig. 2B).

The dose dependency of the effect on blood leukocytes became evident when mice injected once daily for 3 days with MDP were investigated 24 h after the last injection (Fig. 3). Saline-injected controls showed normal leukocyte counts and differentials. Lymphopenia occurred with doses between 30 and 300 mg of MDP per kg, but lymphocytosis was clearly seen at doses of 1 and 3 mg of MDP per kg. Mature neutrophils were apparently not affected (Fig. 3). In view of the very small number of stab neutrophils and monocytes in the blood and to detect potential changes in differentials, the results from blood

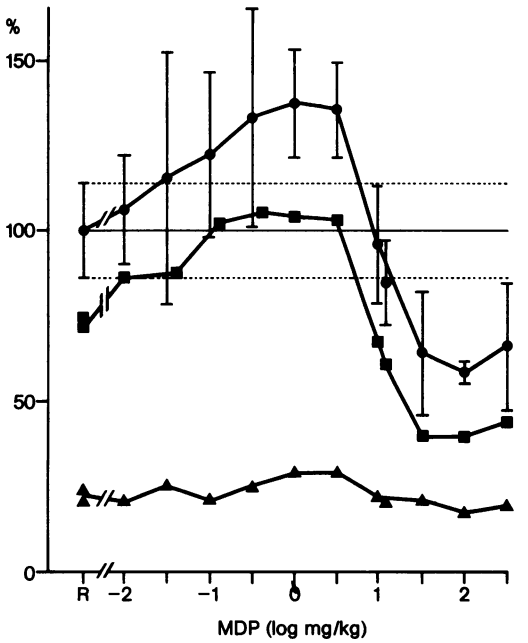

FIG. 3. Dose response of blood leukocytes to MDP 24 h after the last of three daily injections. Abscissa: MDP injected (log of dose in milligrams per kilogram per day; R = nontreated mice; leukocytes 12.3 ± 1.6 × 10^6/ml); ordinate: cell count in percentage of leukocyte counts of corresponding saline controls (●), and neutrophil (▲) and lymphocyte (■) portions of the normalized leukocyte counts. Means and standard deviations (vertical bars) for five mice are given.

cell smears of animals treated with MDP were grouped by MDP dose ranges (Table 1). The number of stab neutrophils and monocytes tended to increase in mice treated with more than 0.3 mg of MDP per kg (Table 1), as shown by the frequency of occurrence in individual mouse smears and the mean percentage of occurrence within one group. This tendency appeared to be largest for stab neutrophils in the low and middle dose ranges and for monocytes in the middle and top dose ranges. The data indicate a dose-dependent increase of lymphocyte, juvenile neutrophil, and monocyte output into circulation, and a disappearance in the periphery, the latter becoming predominant when the dose of MDP exceeds 10 mg/kg.

The number of cells in the peritoneal cavity and the spleen of mice did not change within 48 h of injection of 1 and 100 mg of MDP per kg and of saline alone. However, the cell volume of the peritoneal cells increased by 10 to 15% within 48 h after injection of MDP, and the mononuclear cells appeared to have a larger number of vacuoles, possibly indicating cell activation.

Number and size of BMC after MDP injection. The number of cells harvested from femurs of mice at any time and MDP dose did not change significantly (see Table 4), in contrast to the cell sizes, which did increase (Fig. 4). No difference was seen in the small cell fraction (peak I of the size distribution), which consisted mainly of lymphocytes and blast cells (6). However, a shift toward larger sizes was seen in the large cell fraction (peak II), consisting normally of neutrophils, monocytes, and their late precursors. Such a shift in the mean volume of peak II to the right was larger after repeated injections of MDP (Fig. 4B) and was also dependent on the dose of MDP (Fig. 4C). The morphology of the BMC indicated no change in neutrophil counts but a slight dose-dependent tendency to increased relative counts of large blastic cells and neutrophilic cells with ring-shaped nuclei.

Bone marrow CFU-C counts after MDP injection. Within 24 h of injection of one dose of MDP, the concentration of granulocyte-macrophage progenitor cells in the bone marrow (CFU-C) increased up to 1.5 times that of CFU-C in nontreated mice and mice treated with

TABLE 1. Effect of MDP injection on blood stab neutrophil and monocyte counts[a]

Treatment	No. of mice	Stab neutrophils			Monocytes		
		Sum of counts from *n* mice	Mean counts per animal	% of animals with at least one count	Sum of counts from *n* mice	Mean counts per animal	% of animals with at least one count
None	15	16	1.07	47	7	0.47	27
Saline	34	38	1.12	58	8	0.23	23
MDP (dose: 0.01, 0.03, and 0.1 mg/kg)	15	21	1.40	73	6	0.40	27
MDP (dose: 0.3, 1.0, 3.0, and 10.0 mg/kg)	25	47	1.88	76	40	1.62	80
MDP (dose: 30, 100, 300, and 1,000 mg/kg)	20	22	1.05	45	29	1.45	60

[a] The sum of stab neutrophils and monocytes derived from differentials of 100 leukocytes per animal and a mean count of these cells per mouse were calculated from the sums. The percentage of mice with one or more of indicated cells per 100 leukocytes in their blood is also given.

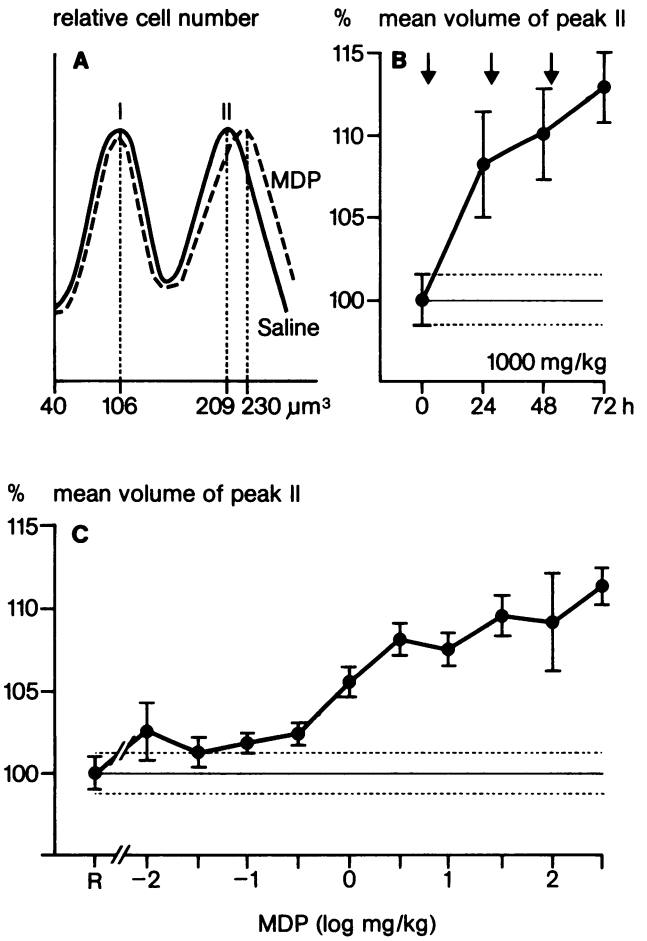

FIG. 4. Effect of MDP on the volume of BMC. (A) Relative size distribution of BMC of mice either untreated (—) or injected subcutaneously with saline (—) or with MDP (---) as determined with a Coulter channelyzer coupled to a Coulter counter. Note the shift of peak II toward a larger size. (B) Effect of repeated injections 1,000 mg of MDP per kg on the mean volume of cells of peak II. Abscissa: hours after injection; ordinate: mean volume of the cells in peak II in percentage of controls; mean values and standard deviation (vertical bars) for five mice are given. Arrow indicates injection. (C) Dose dependency of the volume of the cells 24 h after the last of three daily MDP injections. Abscissa: MDP injected (log of dose in milligrams per kilogram per day; R = nontreated mice); ordinate, as in (B).

saline (Fig. 5), the response of 10 and 100 mg of MDP per kg being of the same order. No such rise in CFU-C was detectable before 8 h after injection of any MDP dose (Fig. 5, inset). It persisted longer as the dose was increased (Fig. 5).

The number of CFU-C increased slightly further over controls in animals injected once daily for 3 days. Because of a smaller variation in the data and a good reproducibility, the 3-day application schedule is more suitable for determining dose responses. An approximately linear relationship was seen for doses of up to 1 mg of MDP per kg, followed by a leveling-off in response for doses from 1 to 10 mg of MDP per kg (Fig. 6). The 50% effective dose was found to be around 0.1 mg of MDP per kg. A double-log plot of relative CFU-C counts versus MDP dose showed a sharper initial increase and a slightly higher plateau level for mice injected once daily for 3 days than it did for mice receiving 1 injection only (Fig. 6, inset). Such an increase in the number of colonies was only seen in vivo, whereas the addition of up to 1 mg of MDP per kg to culture media had no effect (26). In addition to the routinely used procedure of counting nonstained colonies, dishes were stained for aminopeptidase activity (24, 25), thus permitting one to distinguish between granulocyte and mononuclear cell colonies and to determine the

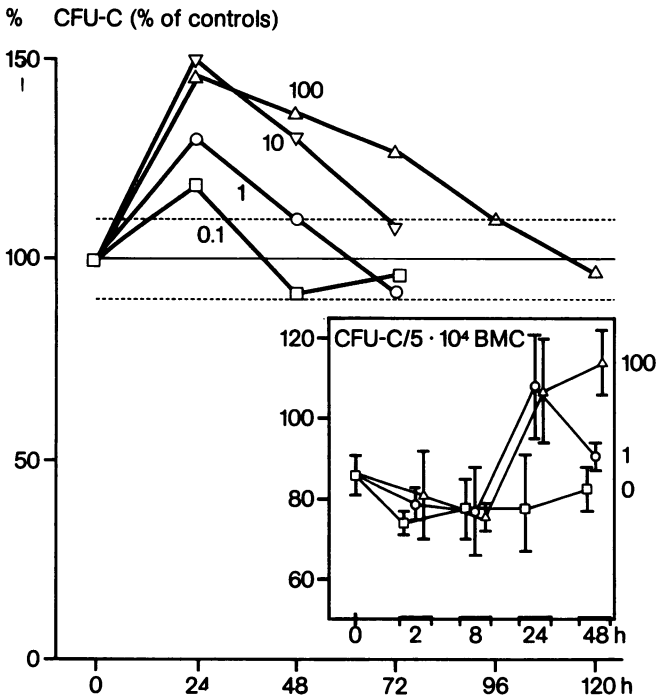

FIG. 5. Bone marrow CFU-C after a single MDP injection. The means and standard deviations for five to ten mice after subcutaneous MDP injections of 0 (0 h), 0.1 mg/kg (□), 1 mg/kg (○), 10 mg/kg (▽), and 100 mg/kg (△). Abscissa: hours after injection; ordinate: CFU-C in percentage of the corresponding controls. Inset: abscissa, as above; ordinate, CFU-C per 5 × 10^4 cultured BMC.

potential of MDP to shift progenitor cells. The number of aminopeptidase-negative colonies containing only polymorphs (as shown in methylene blue-stained smears) remained the same after MDP injection for any time schedule, whereas the number of aminopeptidase-positive colonies (containing both monocytes and polymorphs or monocytes only) increased up to about 150% of their saline controls (Table 2). Only the monocyte-containing BMC appeared to increase after treatment of mice with 1,000 mg of MDP per ml indicating a shift of progenitor cells or an increased rate of differentiation to monocytes.

To investigate the increases of leukocyte progenitor cells in the peritoneal cavity and the spleen, possibly due to immigration from the bone marrow, cells were harvested 2 to 48 h after one injection of saline, or 1 or 100 mg of MDP per kg and cultured. Cell counts in peritoneal washouts and spleen were not affected. Only 1 to 3 colonies per 10^6 cultured peritoneal cells and 15 to 30 colonies per 10^6 cultured spleen cells were found at any time with either dose, and proliferation of peritoneal and spleen CFU-C thus appears unaffected by MDP.

Comparison of MDP and LPS effects on blood leukocytes, serum CSA and bone marrow. It could be argued that the MDP effect on the bone marrow is due to contamination of the MDP preparation with LPS, which is known to induce leukopenia, and the appearance of CSA in serum and CFU-C in the bone marrow (16, 17). Samples of all MDP solutions used were investigated by the *Limulus* amoebocyte endotoxin test and compared with dilutions of standard LPS solutions (Table 3). From this assay it was found that 100 mg of MDP contained less than 5 ng of LPS. On the other hand, investigations of LPS in vivo show a response pattern which is unlike that of MDP. Subcutaneous injections of 0.5 μg of LPS (i.e., more than 10 times the dose of LPS that may contaminate the largest employed dose of 1,000 mg of MDP per kg), neither provoked leukopenia (Table 4) nor a change in femural BMC counts, but provoked a slight dose-dependent increase in the concentration of CFU-C in the bone marrow.

Intraperitoneal injection of a large dose of LPS (500 μg/kg) provoked leukopenia, reduced BMC counts (concomitant with a decrease of cells in size distribution fraction II). It did not change the number of CFU-C per femur, although the CSA content in the serum of such treated mice was large in contrast to that found in mice treated with lower doses of LPS and a

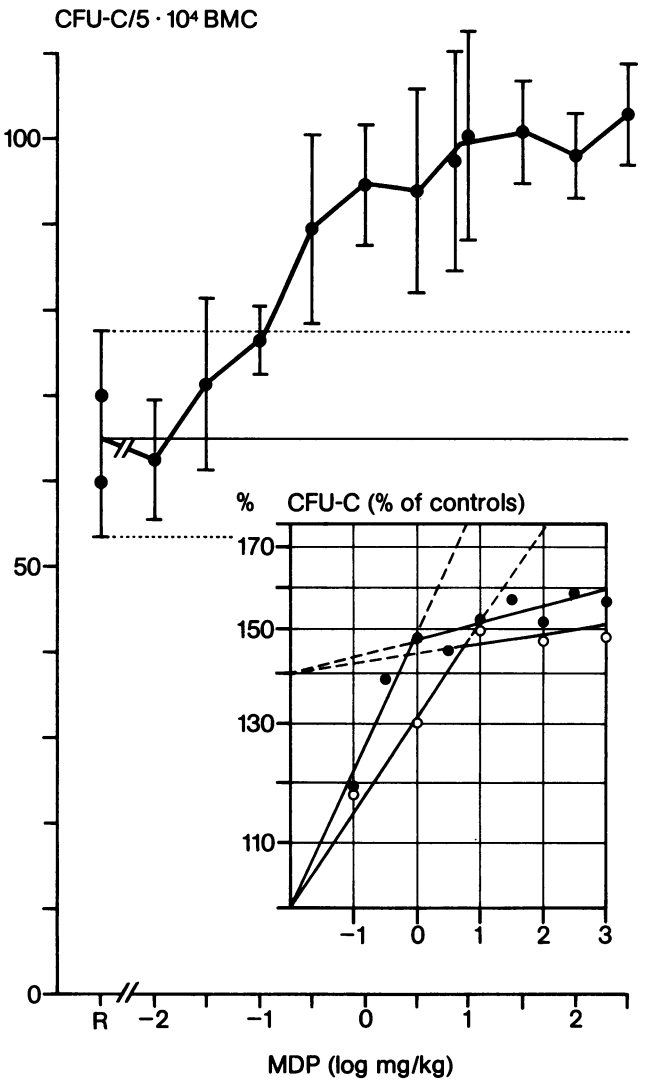

FIG. 6. Dose-response relationship between MDP and bone marrow CFU-C. Abscissa: MDP injected (log of dose in milligrams per kilogram per day; R = nontreated mice); ordinate: CFU-C per 5×10^4 cultured BMC harvested 24 h after the last of one daily injections for 3 days. Mean values for R from two experiments done at different times are given to indicate the range of normal CFU-C counts. Inset: Relationship of dose-response curves after one (open symbols) and three injections (closed symbols, values taken from above). Abscissa: as above; ordinate: CFU-C in MDP-treated mice in percentage of the corresponding saline-treated mice, plotted on a logarithmic scale.

large dose of MDP (Table 4). Therefore, the response of MDP is not due to contamination with LPS.

Stimulation of the immune response to BSA by MDP. MDP is known to stimulate the humoral immune response against various antigens (5). It enhances the production of humoral antibody in mice injected intraperitoneally with a very low dose of BSA. Whereas BSA alone led to no detectable antibody production, BSA injected at the same time as MDP led to an MDP-dose-dependent antibody production (Fig. 7). A similar MDP-dependent-dose response was found when, in addition, MDP was injected once a day on the 2 days subsequent to BSA administration. The stimulation of the humoral response correlated well with the stimulation of granulocyte-macrophage progenitor cells (CFU-C) in the bone marrow (Fig. 8; $r = 0.98$; $n = 5$; $P = 0.001$). However, when MDP was injected daily for 2 subsequent days before BSA and simultaneously with MDP, the humoral response was negligible.

The effect of LPS on the humoral response

TABLE 2. Effect of MDP on bone marrow CFU-C

No. of injections	Subcutaneously injected solution	No. of colonies per 10^4 BMC		
		Total	AP positive	AP negative
0	None	35 ± 3	31 ± 4	4
1	Saline	37 ± 2	33 ± 2	4
	MDP	44 ± 10 ($P < 0.25$)	39 ± 9 ($P < 0.2$)	5
2	Saline	28 ± 4	24 ± 5	4
	MDP	53 ± 8 ($P < 0.001$)	47 ± 7 ($P < 0.001$)	6
3	Saline	32 ± 8	26 ± 9	6
	MDP	55 ± 5 ($P < 0.001$)	51 ± 6 ($P < 0.001$)	4

[a] Mice were once daily injected with 1000 mg of MDP per kg. BMC were harvested 24 h after the last injection and treated by a hypotonic shock for 30 s to lyse erythrocytes and thus to reduce the number of cells in the culture. After 7 days of culturing, the number of colonies per dish was counted before and after staining for aminopeptidase (AP) activity. Means and standard deviations of five MDP-treated mice, five nontreated mice, and three saline-treated mice are given. The significance of difference from each saline group was determined.

was tested by once injecting LPS simultaneously with BSA. Antibody against BSA was not detectable with 0.1 μg/kg and smaller doses.

Plateau levels of antibody titers, similar to MDP, were achieved with 100 μg of LPS per kg and larger doses. On the other hand, plateau levels were reached with 10 mg of MDP per kg which, at the most, would be equivalent to 0.5 ng of LPS per kg. Therefore, the response to MDP is not due to a potential LPS contamination.

Response to a pharmacologically inactive MDP derivative. The MDP analog 2-acetamido-2-deoxy-3-0-[(L-1-(D-1,3-bis-methylcarbamoyl-propyl)-carbamoylethyl)-carbamoylmethyl]-D-glucose, injected at doses up to 300 mg/kg simultaneously with BSA, did not stimulate the immune response. The potential contamination with LPS was less than 5 ng of LPS in an 100-mg test compound as determined by the *limulus* amoebocyte endotoxin test. The number of CFU-C in mice 24 h after injection of 100 mg of this analog per kg was 84 ± 10 per 5×10^4 bone marrow cells, compared with 83 ± 7 for saline and 114 ± 8 colonies for mice treated with 100 mg of MDP in the same experiment.

TABLE 3. Titration of LPS with the *Limulus* amoebocyte test

Treatment	Test results (after given time of: [min])		
	15	30	60
0.9% Saline	–	–	–
LPS (500 ng/ml)	ND[a]	+	ND
LPS (50 ng/ml)	+	+	+
LPS (5 ng/ml)	+	+	+
LPS (0.5 ng/ml)	–	+	+
LPS (0.05 ng/ml)	–	–	±[b]
MDP (125 mg/ml)	–	+	+
MDP (12.5 mg/ml)	–	–	±

[a] ND, Not done.
[b] ±, Viscous, not firm gel.

DISCUSSION

Three major dose-dependent effects have been observed after subcutaneous injection of MDP in mice: lymphocytopenia in the blood followed by lymphocytosis, an increase of monocyte progenitor cell counts in the bone marrow with a concomitant relative monocytosis in the blood, and stimulation of the humoral immune response. One explanation for these phenomena is a response of the macrophage system mediated by MDP. CFU-C in the bone marrow may rise as a direct effect of MDP on myeloid progenitor cells or, alternatively, as an effect on an as-yet-unidentified affector cell, which in turn stimulates recruitment or proliferation of myeloid progenitor cells. As a consequence of stimulated proliferation, CFU-C-derived monocytes will increase, and thus more macrophages will become available to handle the antigen, thereby regulating humoral immune response and resistance to infection (2, 9).

When MDP was injected at the same time as the antigen and after antigen administration, the immune response was stimulated (Fig. 7). The effect of the MDP dose on the immune response correlated well with the increase in bone marrow CFU-C counts (Fig. 8). The finding is compatible with the explanation that MDP may provide more cells for antigen presentation to immunocompetent cells, since the monocyte CFU-C (Fig. 5 and 6) and the blood monocytes (Tables 1 and 2) increase while, presumably, enough antigen is still present in the body. However, in agreement with previous reports (5, 10), administration of MDP before the antigen does not stimulate the immune response (Fig. 7), although increased resistance to infection under these conditions has been reported (2, 7, 9). This finding is not unexpected in view of the activation of macrophages by MDP and the dependency of any immune response on the dose of the antigen. Activation of macrophages has been

TABLE 4. Comparison of the effects of MDP and LPS on blood leukocytes, serum CSA, BMC count, and CFU-C[a]

Treatment	Blood		Bone marrow		
	Leukocytes (10^6/ml of blood) at 4 h	Serum CSA (colonies/5 × 10^4 BMC) at 4 h	Cellularity (10^6 cells/femur) at 24 h	CFU-C (colonies/5 × 10^4 BMC) at 24 h	CFU-C (10^4 colonies/femur) at 24 h
0.9% saline	12.0 ± 1.9	1–2	14.0 ± 3.1	84 ± 8	2.3 ± 0.3
MDP, subcutaneous (100 mg/kg)	5.7 ± 0.6 ($P < 0.025$)	<5	12.4 ± 1.3	134 ± 9	3.5 ± 0.4 ($P < 0.005$)
LPS, subcutaneous (0.5 μg/kg)	11.3 ± 4.5	2	14.0 ± 1.3	98 ± 8	2.8 ± 0.4 ($P < 0.1$)
LPS, subcutaneous (5 μg/kg)	12.4 ± 0.7	2	11.4 ± 3.3	114 ± 8	2.4 ± 0.7 ($P = 0.4$)
	at 2 h	at 2 h	at 24 h	at 24 h	at 24 h
0.9% Saline	13.5 ± 0.7	1	13.4 ± 1.1	84 ± 11	2.2 ± 0.2
LPS, intraperitoneal (500 μg/kg)	4.1 ± 1.1 ($P < 0.001$)	62	9.9 ± 2.1 ($P < 0.005$)	103 ± 7	2.0 ± 0.5 ($P < 0.3$)

[a] Four mice were analyzed per dose group. CSA determinations were done in duplicates of sera pooled from these four mice. The significance of difference from saline controls was determined.

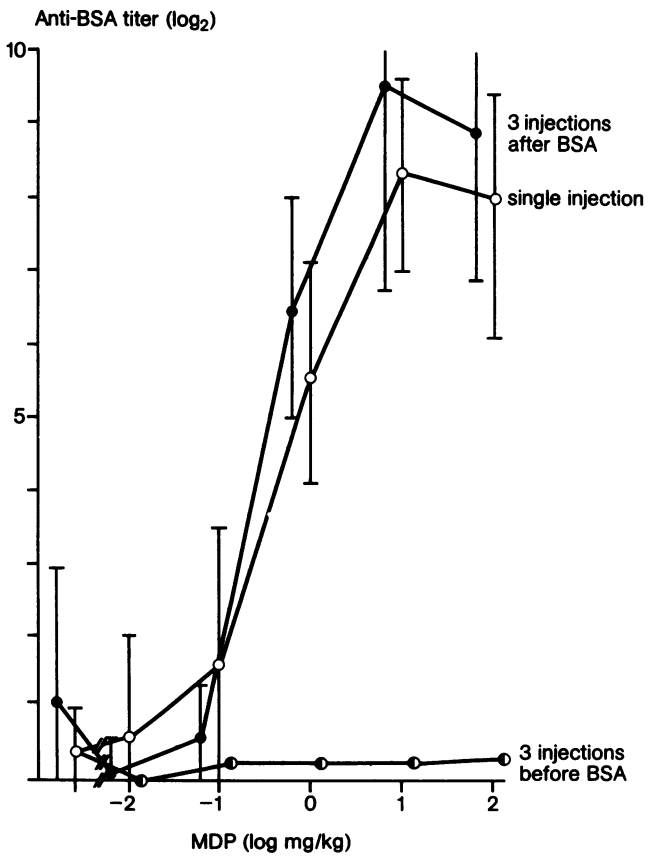

FIG. 7. Anti-BSA antibody titers against a very low dose of BSA after MDP injection. Mean titers and standard deviations for eight mice measured 27 days after one intraperitoneal injection of 10 μg of BSA. MDP was injected subcutaneously once simultaneously with BSA (○), once daily for two days before BSA and simultaneously with BSA (◐), and once daily simultaneously with BSA and on the 2 subsequent days (●). Abscissa: dose of MDP per day (log of dose in milligrams per kilogram); ordinate: Anti-BSA titers in serum (expressed as $\log_2$ of hemagglutination titers).

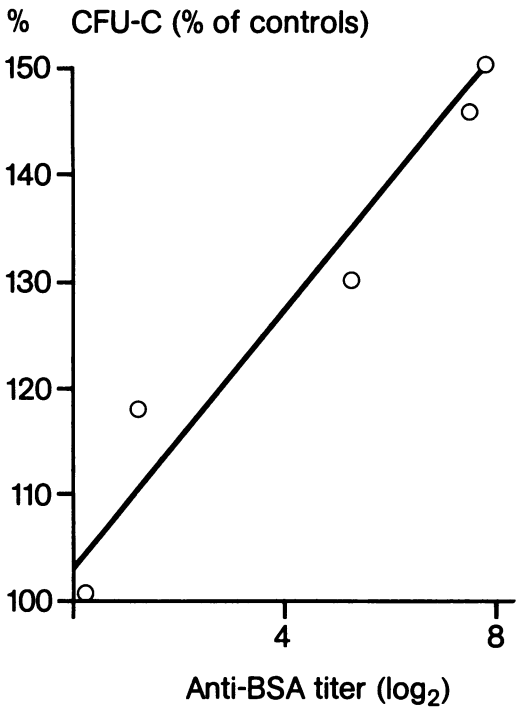

FIG. 8. Correlation of increases in CFU-C number with the enhancement of the humoral response after (toward BSA) one MDP injection. CFU-C counts were determined 24 h after MDP injection. Immunization was performed at the time of MDP injection. Abscissa: Anti-BSA titers in serum (expressed as $\log_2$ of titers); ordinate: CFU-C counts in percentage of corresponding controls.

shown both in vitro (16) and in vivo (2) and resulted in enhanced clearance rates (22, 28). Therefore, administration of MDP provokes an increase in macrophages by a proliferative response of precursor cells in the bone marrow and an activation of these cells. Consequently, antigen or infectious agents administered after the MDP may be removed rapidly, and only little or no antigen remains for the immune competent cells to mount an immune response.

Although blood neutrophil counts do not change after injection of MDP, the dose-dependent increases in the counts of both stab neutrophils and monocytes in the blood (Table 1) and the CFU-C in the bone marrow (Tables 2 and 4) indicate an increased output of cells from the marrow into the periphery. By contrast, progenitor cells are not increased in the spleen and peritoneal cavity. Interestingly, the slight dose-dependent lymphocytosis 24 h after MDP administration (Fig. 3) parallels the doses leading to increases in CFU-C formation (up to a plateau). These low-dose lymphocytotic responses might be the result of an increase in lymphocyte proliferation. On the other hand, lymphocytopenia is observed within 24 h of one injection and persists for more than 24 h after three injections, but only at doses above 10 mg of MDP per kg, i.e., doses which give rise to plateau levels of CFU-C in the bone marrow. Thus, although lymphocytopenia is dose-dependent, it does not appear to be related either to the immune response or to bone-marrow cell proliferation and might, therefore, be considered an adverse effect.

It is conceivable that the rise in bone marrow progenitor cells is induced by CSA released from macrophages after MDP injection. That such activation is possible has been demonstrated in vitro with murine peritoneal macrophages (20). However, the amount of CSA found in the serum of mice after injection of MDP is minimal, even with large doses of MDP, in contrast to LPS (17), which induces the release of CSA but no increase in CFU-C (Table 4). This finding indicates that CSA from macrophages plays only a minor role. On the other hand, MDP is unlikely to have a direct effect on CFU-C in vivo, since, in contrast to bestatin, another well-known immunomodulator, MDP does not enhance growth of colonies in vitro (26). Nevertheless, the increased pool of CFU-C in the bone marrow could be the expression of an effect of MDP on the recruitment of committed stem cells leading to a preferential increase of mononuclear cells (Table 2). LPS may increase the rate of proliferation of CFU-C, without an effect on the CFU-C counts per femur, but with a decrease in bone marrow cellularity after an increase in blood CSA (Table 4) (4, 12).

These distinct differences in their stimulating properties apparently reflect the structural composition of the two bacterial-cell-wall-derived components: LPS is a lipopolysaccharide with a molecular weight of several thousands and covalently linked to lipid-A (4), whereas MDP is a low-molecular-weight peptidoglycan without lipid-A (13).

In conclusion, the good correlation between immune response and progenitor cell proliferation suggests that the regulation of the numbers and the activity of macrophages play a major role in the stimulation of the immune response by MDP.

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