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Original Scientific Paper

Alteration of Lysosomal Membrane and Enzymes Induced by Peptidoglycan Monomer (PGM)

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The influence of single (4 mg/mouse) and multiple (1 mg/mouse/day for 5 consecutive days) injections of PGM on some hepatic enzymes, lipid peroxide generation in serum and liver and hepatic lysosomal membrane permeability was investigated. The studies performed showed that a single injection of PGM *in vivo* changed temporarily the permeability of lysosomal membranes, lipid peroxidation products and, when administered *in vitro*, modulated superoxide anion production and did not affect the activity of lysosomal membrane enzymes. Multiple injections of PGM caused no significant changes in the examined parameters. Although the metabolic changes were time-limited and, from the toxicological point of view, provoked transient effects, the results obtained may be of importance when using PGM in combined chemo-immunotherapy.

INTRODUCTION

Peptidoglycan monomer (PGM), a constituent of bacterial cell walls, has various biological activities: antitumor and antimetastatic activity *in vivo*,[1] inhibition of tumor growth and spreading of metastases in melanoma B-16[2] and stimulation of humoral and cellular immune reactions.[3] It is a water soluble, apyrogenic and non-toxic compound. However, undesired side effects

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of PGM treatment on the activity of important drug-metabolizing enzymes or intracellular lytic bodies, lysosomes, are possible. Namely, many pharmacologically active substances may damage the integrity and change the function of lysosomal membranes. It has been repeatedly shown that such damage may either lead to a number of pathological conditions[4] or be useful in therapy.[5] Therefore, the aim of this work was to investigate the effects of PGM treatment on the activity of lysosomal enzymes, lysosomal membrane permeability and lipid peroxide generation. Hepatic cellular damage was monitored by measurements of cytoplasmic lactate dehydrogenase.

EXPERIMENTAL

Chemicals

PGM, GlcNAc-MurNAc-L-Ala-D-*iso*glutamine-*meso*- diaminopimelic acid-D-Ala-D-Ala, was obtained as previously described.[6] The PGM preparation used in this study contained less than 0.1 IU of endotoxin per 1 mg of PGM, as determined by the Q.C. LAL assay (Bio-Whitaker, USA). Triton X-100, Ferricytochrome C Tipe III, 1,1,3,3-tetramethoxypropane, *N*-formyl-L-methionyl-L-phenylalanine and bovine serum albumin were purchased from Sigma (St Louis, Mo, USA) and phorbol myristate acetate from the Consolidated Midland Co., (Brewster, NY). *N*-acetylneuraminic acid was obtained from Boehringer (Mannheim, Germany). The substrates for enzyme assays were purchased from Koch-Light Laboratories Ltd (Colnbrook, Bucks, UK). All other chemicals were of the highest purity obtainable.

Animal experiments

Male CBA/HZgr mice, three months old, weighing 25–30 g, from the breeding colony of the Ruđer Bošković Institute were used. They were kept four to a cage on a standard diet with food and water given *ad libitum*. Since intraperitoneal, like intravenous or subcutaneous, administration of PGM resulted in a significant stimulation of the immune response, the animals were injected PGM i/p, either in a single dose of 4 mg/mouse, or in multiple doses of 1 mg every 24 hrs for 5 days. For each time interval tested, control mice that were given saline only were used. This experimental design and the doses given were selected on the basis of previous observations which showed that only doses larger than 0.5 mg/mice of PGM affected the liver enzyme activities, citochrome P-450 content[7,8] and reduced the number of lung metastases in melanoma B-16, Lewis lung carcinoma and mammary carcinoma bearing mice.[2,9]

The mice injected with a single dose of PGM were sacrificed 1, 12, 24 or 48 hours after injection and those treated with multiple injections 24 hrs after the last PGM administration. Only in experiments where the β-glucosidase activities (BG) were determined, the mice were sacrificed 3, 24, 48 and 78 hours after single PGM injections. The livers were removed immediately, blotted on filter paper and weighed. For the assay of lipid peroxides formation, the livers were first washed in ice-cold saline and then liver homogenates were prepared in a ratio of 1 g of wet tissue to 9 ml of 1.15% KCl by using an ice-jacked Potter- Elvehjem homogenizer (1300 rev/min). Tissue preparation, test for the *in vitro* effect of PGM on the activities of lysosomal hydrolase (acid phosphatase, AP and BG) and test for the *in vivo* effect of PGM on mice liver lysosomes were prepared as described by Šverko *et al.*[10]

Biochemical assays

AP (EC 3.1.3.2.) in the liver was measured by the method of Berthet *et al.*[11] using sodium β-glycerophosphate as a substrate hydrolyzed by lysosomal AP, and in the serum according to the method of Štraus.[12] BG (EC 3.2.1.2.) was assayed as reported by Beck and Tappel[13] and lactate dehydrogenase (EC 1.1.1.27) by the method of Wacker *et al.*[14] Specific enzyme activities were defined as μmoles of products formed per mg of protein, per g of fresh liver per min. or as U/L. Superoxide anion formation (O_2^-) by mouse macrophages was studied by the technique based on superoxide dismutase (EC 1.15.1.1.)-sensitive reduction of ferricytochrome C to ferrocytochrome C.[15] O_2^- production by macrophages was initiated with phorbol myristate acetate (1μg/ml) or with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (5×10^{-6} M) added to the incubation medium. The effects of PGM concentrations of 10, 100 and 1000 μg/ml were assayed. Protein concentration in the samples was measured by the method of Lowry *et al.*[16] using bovine serum albumin as standard. Lipid peroxide formation was estimated estimated according to the presence of thiobarbituric acid reactive substances (TBARS) in the liver[17] and plasma.[18] The procedure measures several aldehydic products of lipid peroxidation, mainly malondialdehyde.

Statistical analysis

The data are presented as mean values ± standard deviation. The significance of the differences was assessed by Student's *t*-test. The P values < 0.05 were considered significant.

RESULTS

Effect of in vivo PGM treatment on liver lysosomes and lipid peroxidation

A significant loss of unsedimentable AP activities in mice livers was observed as early as 12 hrs after a single injection of 4 mg of PGM (Figure 1A). The effect persisted up to 24 hrs after PGM administration. This decrease of AP activities in liver was accompanied by low serum AP levels, but only 12 hrs after the treatment with 4 mg of PGM (Figure 1B). Treatment with 4 mg of PGM did not influence the BG activity measured 3, 24, 48 and 72 hours after injection (Figure 2). However, there was a significant ($P<0.01$) decrease in the activity of this enzyme 48 hours after administration of the same dose of PGM.

Treatment of mice with 4 mg of PGM remarkably affected also the level of lipid peroxidation products 24 hrs after administration, *i.e.* significantly increased the TBARS concentration in the plasma (Figure 3A) and decreased the TBARS concentration in the liver (Figure 3B).

Cytoplasmic lactate dehydrogenase activity was slightly, but statistically insignificantly, reduced in PGM-treated mice 24 hrs following the treatment (23.06 ± 2.21 U/L *versus* 24.76 ± 1.72 U/L in the controls).

Multiple injections of PGM (1 mg for 5 consecutive days) caused no significant changes in the TBARS concentration in the liver (control 115.24 ± 12.54

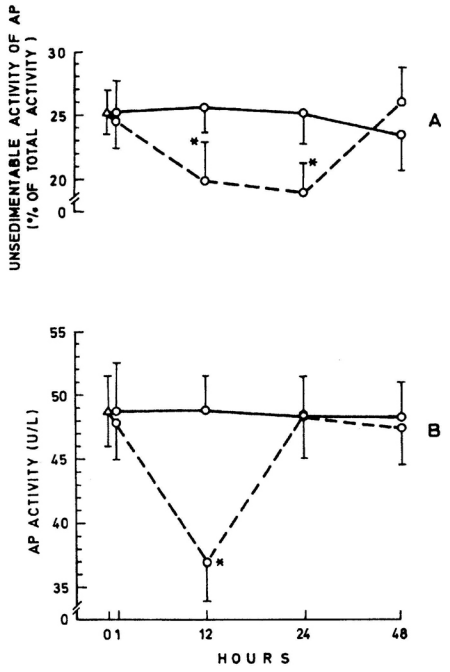

Figure 1. Unsedimentable acid phosphatase activities in the whole liver homogenates (A) and acid phosphatase activities in the serum (B) of mice after a single injection of 4 mg of PGM at different time intervals. Each point represents the mean ± SD of three separate experiments (four mice per point for each experiment). Δ untreated controls, o——o saline treated controls, o - - - o PGM-treated mice, $^{*}P < 0.001$ *versus* saline-treated controls.

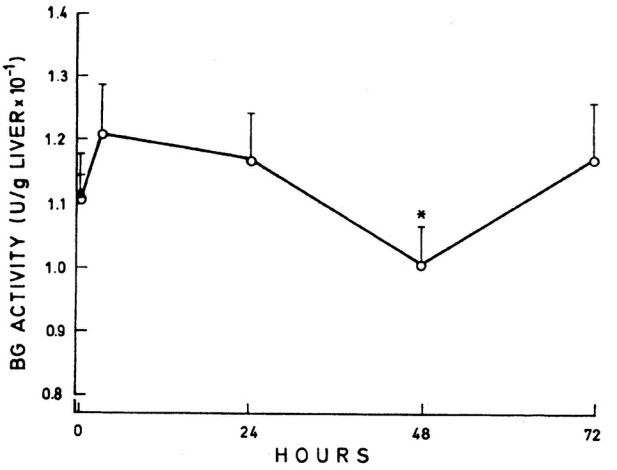

Figure 2. The β-glucosidase activities in the whole liver homogenates of mice after a single injection of 4 mg of PGM at different time intervals. Each point represents the mean ± SD of three separate experiments (four mice per point for each experiment). Δ untreated controls, o saline-treated controls, o——o PGM-treated mice, $^{*}P < 0.01$ *versus* saline-treated controls.

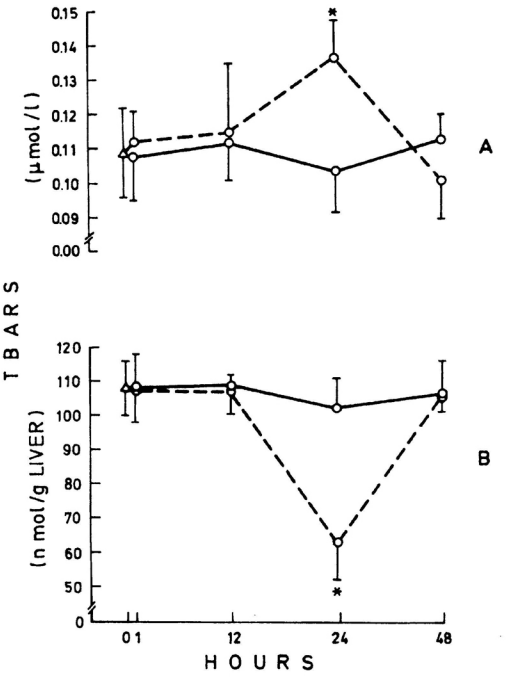

Figure 3. Lipid peroxidation level in the plasma (A) and liver (B) of mice after a single injection of 4 mg of PGM at different time intervals. Each point represents the mean ± SD of three separate experiments (four mice per point for each experiment). Δ untreated controls, o——o saline-treated controls, o - - - o PGM-treated mice, $^{*}P < 0.001$ *versus* saline-treated controls.

nmol/g liver, PGM 109.26 ± 8.40 nmol/g liver) and plasma (control 0.105 ± 0.009 µmol/L; PGM 0.110 ± 0.014 µmol/L). Since there were no significant differences between the other parameters studied in the control and PGM treated mice, they are only reported without graphical presentation.

Effects of in vitro PGM treatment upon the activities of lysosomal enzymes and superoxide anion release (O_2^-)

When the liver lysosome-rich suspensions were incubated with PGM for 10 min at 37 °C, significantly increased AP activities were observed only at concentration of 1000 µg/ml. The BG activities of liver lysosome-rich suspensions were not altered by any PGM concentration tested (Table I).

O_2^- release from mouse macrophages was suppressed with a low concentration of PGM (10 µg/ml). Otherwise, superoxide anion release was stimulated (Figure 4) at higher concentrations of PGM (100 or 1000 µg/ml).

DISCUSSION

The present data show that PGM induced time-dependent inhibition of enzyme release from liver lysosomes. This may suggest that PGM interferes with the function of the liver lysosomal membrane. At present, it is not clear

TABLE I

Specific activities of acid phosphatase and β-glucosidase after incubation with PGM for 10 min at 37 °C expressed as μmoles of the product released/min/mg protein. Each value represents the mean ± SD of three independent experiments, four mice per point per each experiment. $^{*}P < 0.02$ *versus* controls.

PGM (μg/ml)	Acid phosphatase	β-glucosidase
Control	26.18 ± 1.65	10.19 ± 0.83
0	26.18 ± 1.85	10.87 ± 1.59
250	26.18 ± 1.65	8.73 ± 2.05
500	32.84 ± 4.36	10.30 ± 1.72
1000	41.41 ± 4.95*	8.94 ± 1.66

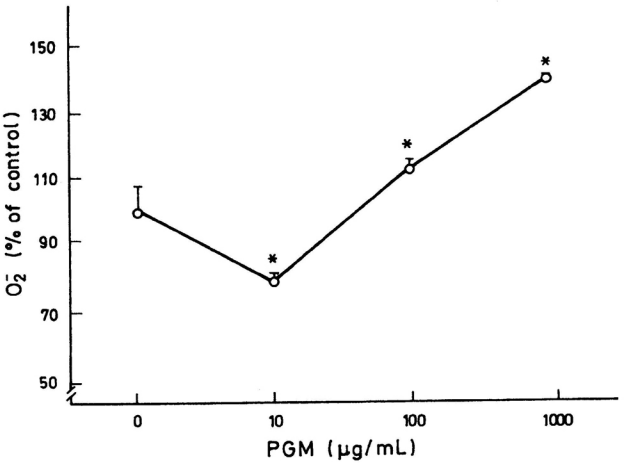

Figure 4. Effect of PGM O_2^- upon release by mouse macrophages. Values of O_2^- release from PGM treated macrophages are expressed in % of the control values. Each value represents the mean of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* saline-treated controls.

whether such interference is a result of: a) membrane stabilization, b) indirect interaction of PGM with the activity of membrane bound enzymes, or c) a combination of both possibilities. Direct interaction between PGM and lysosomal enzymes seems unlikely, because addition of PGM to lysosome-rich suspensions *in vitro* induced no significant changes in the AP and BG activities. An effect was recorded of an especially high dose of PGM (1000 μg/ml) on the AP activity ($P < 0.02$) in lysosome-rich suspensions. Reduced unsedimentable activities of AP in whole liver homogenate were paralleled by decreased serum AP activities and decreased BG activities in the liver. There are reports in the literature on the inhibitory effect of PGM on the microsomal

cytochrome P-450 system[7] and diminished hepatic microsomal UDP-glucuronyltransferase and β-glucuronidase[8] in mice. Glycosidases are present both in microsomes and lysosomes. They are precursors of the mature lysosomal enzyme.[19] Therefore, the inhibitory action of PGM on the microsomal enzyme may influence the activity of lysosomal enzymes. According to the reports of Renton *et al.*[20] or Williams and Szentivanyi,[21] the inhibition of liver drug- metabolizing enzymes may be related to the enhancement of the release of certain endogenous inhibitors after administration of imunoactive drug. In rats treated with carbon tetrachloride, administration of an analogue of muramyl dipeptide, a muramyl peptide very similar to PGM, reduced the high serum levels of alanine- and aspartate transaminases, lactate dehydrogenase and cytosolic enzymes.[22] Balitsky *et al.*[23] have shown that the 5'-nucleotidase activity in murine macrophages *in vitro* decreases after exposure to glucosaminyl muramyl dipeptide. These reports suggest that immunomodulators act by modulation of superoxide anion production, which results in an inhibition of the agents metabolizing enzymes. Our results seem to support this concept since PGM, in concentration of 100 μg/ml or higher, stimulates superoxide anion formation also *in vitro*. Similarly, Simčić *et al.*[24] and Kaku *et al.*[25] have shown that muramyl dipeptide and some of its derivatives induce superoxide anion release from mouse peritoneal macrophages *in vitro* and *in vivo*.

Plasma TBARS concentration increased significantly 24 hrs after administration of 4 mg of PGM. The increase of TBARS in plasma may be attributed to PGM-stimulated macrophages, the cells to which PGM preferentially binds.[26] Alternatively, the PGM induced elevation of TBARS concentration in plasma could be a result of increased plasma iron and coopper levels shown to occur after treatment with a muramyl dipeptide analogue.[22] These metal ions are active catalysts[27] and stimulate the formation of lipid peroxides.

Unlike in the plasma, the TBARS concentration in the liver of PGM-treated mice decreases and parallels the decrease in unsedimentable AP activities from lysosomes. This effect may be related to the stabilization of lysosomal membrane and decreased level of cytochrome P_{450}. Namely, disorganization of the lysosomal membrane, lipid peroxide formation and enzyme release are closely related.[28,29] Also, cytochrome P_{450} is known to act as a source of new radicals and its diminished content in the liver following the PGM treatment[7] might contribute to the decrease in TBARS levels. In our experimental conditions, no influence of PGM upon the release of cytoplasmic lactate dehydrogenase could be observed.

Although the alteration of the function of lysosomal membranes and release of lipid peroxide are only transient, these findings are of importance in planning a rational use of PGM administration in the treatment of immunodefficiences or cancer.

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ABBREVIATIONS: PGM, peptidoglycan monomer; AP, acid phosphatase; BG, β-glucosidase; TBARS, thiobarbituric acid reactive substances.

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SAŽETAK

Promjene lizosomske membrane i enzima izazvanih peptidoglikan-monomerom (PGM)

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Istražen je utjecaj jednostruke (4 mg/miš) i višestruke (1 mg/miš/dan tijekom 5 dana) primjene PGM-a na aktivnost nekih jetrenih enzima, stvaranje lipidnih peroksida u serumu i jetri te propusnost membrana lizosoma u jetri miša. Jednostruka primjena PGM-a *in vivo* prolazno je promijenila propusnost lizozomskih membrana, lipidnih peroksida, dok je primjena *in vitro* modulirala nastanak superoksidnih aniona, ali nije utjecala na aktivnost enzima membrana lizosoma. Višestruka primjena PGM nije uzrokovala značajne promjene u promatranim pokazateljima. Iako su metaboličke promjene bile vremenski ograničene i toksikološki gledano, izazivale prolazne učinke, dobiveni rezultati mogli bi biti važni pri primjeni PGM-a u kombiniranoj imuno-kemoterapiji.