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Effect of immunoadjuvant peptidoglycan monomer on liver cytochrome P-450

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Bacterial cell walls contain several components which influence the host's immune response. Peptidoglycans are the typical cell wall constituents possessing versatile biological activity, including immunostimulation [1, 2] and enhancement of tumor defence in experimental animals [3, 4]. Naturally occurring and synthetic low mol. wt peptidogylcan fragments are mostly devoid of toxic effects noticed in treatments with high mol. wt preparations.

Treatment with various bacterial preparations [5–9] produced in experimental animals a decrease in cytochrome P-450 levels and in related enzyme activities. So far, to the best of our knowledge, the effect of low mol. wt peptidoglycans on the hepatic microsomal cytochrome P-450 system has not been reported.

Such studies would be of importance, particularly when applied to combined adjuvant and chemotherapy of cancer; the metabolism of various antitumor agents might be affected by the action of immunostimulators on cytochrome P-450 and related drug metabolizing enzymes.

In this paper, the effect of a chemically well-defined, hydrosoluble peptidogylcan monomer on cytochrome P-450 content and 7-ethoxycoumarin-*O*-deethylase activity following intra-venous administration to mice, was studied. Peptidoglycan monomer, GlcNAc–MurNAc–L-Ala–D-isoglutaminyl–*meso*-diaminopimelyl–D-Ala–D-Ala [10, 11], was shown previously to stimulate the humoral immune response to particulate antigens in mice [12, 13] and to reduce the number of lung metastases in melanoma B-16 and Lewis lung carcinoma [14, 15] bearing mice.

Materials and methods

Peptidoglycan monomer and fragments. Peptidoglycan monomer was prepared by lysozyme digestion of uncross-linked peptidogylcan chains isolated from culture fluids of penicillin treated *Brevibacterium divaricatum* NRRL-2311 as already described [10, 11]. All preparations used were tested for endotoxin content. The disaccharide, GlcNAc–MurNAc, and the corresponding pentapeptide, L-Ala–D-isoglutaminyl-*meso*-diaminopimelyl–D-Ala–D-Ala, were obtained by hydrolysis of peptidoglycan monomer with *N*-acetylmuramyl-L-alanine amidase from human sera [16].

Animals. Female C57Bl mice, weighing 16–20 g, were used throughout. Treated animals received intra-venously, via the tail vein, either 1 mg of peptidoglycan monomer (unless quoted otherwise) or 0.5 mg of the corresponding disaccharide and pentapeptide, respectively, freshly dissolved in 0.9% NaCl (0.2 ml). Controls were given only saline.

Preparation of microsomes. A 25% liver homogenate in 1.15% KCl was centrifuged at 10,000 *g* for 15 min. The supernatant was apllied to a Sepharose 2B column (10 × 200 mm) and eluted with Tris–HCl buffer, pH 7.6 [17]. The milky fraction coming out of the column before the red band of hemoglobin was collected as the one richest in cytochrome P-450 activity. Protein was determined by the method of Lowry *et al.* [18].

Fluorometry. An Aminco-Bowman spectrophotofluorometer was used. The fluorescence of 7-hydroxycoumarin was measured at 465 nm with excitation at 365 nm. 7-Ethoxycoumarin was a kind gift from Professor V. Ullrich.

Assay of cytochrome P-450. The estimation of cytochrome P-450 content was done according to the method of Omura and Sato [19].

7-Ethoxycoumarin-O-dealkylation assay. The method is a modification of the procedure of Ullrich and Weber [20]. The incubation mixture contained in 1.0 ml Tris–HCl buffer, pH 7.6, the following components: 1 mg microsomal protein, 10^{-4} M 7-ethoxycoumarin and 10^{-4} M NADPH. Reaction was initiated by the addition of NADPH and allowed to proceed for 10 min at 30°. The production of 7-hydroxycoumarin was linear within 15 min.

In *in vitro* experiments, the incubation mixture was the same, except that 10^{-4} M peptidogylcan monomer was added.

Statistical procedures. Each group consisted of five mice which were tested individually; all experiments were performed 2–4 times. Student's *t*-test was used to determine the statistical significance of the difference from the controls.

Results and discussion

Treatment of mice with 1 mg (1 μmole) of peptidoglycan monomer was followed by the time dependent decrease in cytochrome P-450 content (Table 1). The effect was most marked after 24 and 48 hr; 72 hr after administration of peptidoglycan monomer, the levels of cytochrome P-450 returned to normal. The delayed action on cytochrome P-450 and the duration of the effect (up to 48 hr) are rather surprising, since it was found that peptidoglycan monomer and its metabolite are excreted almost completely from the organism in a few hours after intra-venous injection [21].

Various doses of peptidoglycan monomer (0.2–4.0 mg) produced the decrease in cytochrome P-450 content, but the effect was not clearly dose related (Table 2). A statistically significant decrease was observed with doses larger than 0.5 mg. It is interesting to note that a similar pattern in dose-response was also observed in immune response to particulate antigens with peptidoglycan monomer as adjuvant [13]. The convenient effective therapeutic dose for treatment of lung metastases in mice was found to be 1 mg per mouse [14, 15] and, therefore, such a dose was predominantly tested in experiments presented in this paper.

Some drugs and biologically active compounds interact with cytochrome as ligands, eliciting the formation of characteristic binding spectra. In *in vitro* experiments, the addition of various doses of peptidoglycan monomer (100–500 μg/ml) to microsomal preparations did not induce any change in the absorbance between 500 and 380 nm. Therefore, it seems unlikely that the decrease in cytochrome P-450 observed in *in vivo* experiments is caused by direct interaction with peptidoglycan monomer.

Studies with the fragments of peptidoglycan monomer (obtained by hydrolysis with *N*-acetylmuramyl-L-alanine amidase) [16] were also conducted to determine whether the decrease in P-450 level could be attributed to peptidoglycan monomer as an integral molecule, or to its disaccharide or pentapeptide portion, respectively. It was found that only pentapeptide (1 μmole) affects significantly the cytochrome P-450 content (Table 3), which is in accord with our findings that the pentapeptide part of the molecule is also responsible for immunostimulating effects in mice (I. Hršak, J. Tomašić and T. Parotti, unpublished results).

The decrease in cytochrome P-450 level was paralleled by a decrease in 7-ethoxycoumarin-*O*-deethylase activity (again 24 and 48 hr after administration of peptidoglycan

Table 1. Effect of peptidoglycan monomer (PGM) on cytochrome P-450 content and the time course of 7-ethoxycoumarin-*O*-deethylase activity

Time after administration (hr)	Cytochrome P-450 content (nmoles/mg protein)		PGM-treated/control (×100)	7-Ethoxycoumarin-*O*-deethylase activity (nmoles/mg protein·min)		PGM-treated/control (×100)
	Control	PGM-treated mice		Control	PGM-treated mice	
3	0.75 ± 0.02	0.82 ± 0.02	109.3	0.78 ± 0.04	0.70 ± 0.06	89.2
24	0.75 ± 0.03	0.55 ± 0.02	73.3*	0.77 ± 0.02	0.53 ± 0.03	68.8†
48	0.75 ± 0.03	0.56 ± 0.03	74.6*	1.06 ± 0.05	0.80 ± 0.07	75.5*
72	0.79 ± 0.03	0.77 ± 0.02	97.5	0.78 ± 0.15	0.77 ± 0.01	98.71
96	0.73 ± 0.02	0.82 ± 0.02	112.3	N.D.	N.D.	—

Mice were treated intra-venously with 1 mg (1 μmole) of PGM in saline. Control groups received only saline. Results are means ± S.E. of 2–4 groups of animals; each group consisted of five mice.

* Significantly different from control values, $P < 0.05$.

† Significantly different from control values, $P < 0.01$.

N.D., Not determined.

Table 2. Effect of various doses of peptidoglycan monomer (PGM) on cytochrome P-450 content after 24 and 48 hrs

Dose of PGM (mg)	24 hr			48 hr		
	Cytochrome P-450 content*		PGM-treated/control (×100)	Cytochrome P-450 content*		PGM-treated/control (×100)
	Control	PGM-treated		Control	PGM-treated	
0.2	0.78 ± 0.07	0.67 ± 0.06	85.9†	0.95 ± 0.05	0.80 ± 0.04	84.2†
0.5	0.78 ± 0.07	0.68 ± 0.06	87.0†	0.95 ± 0.05	0.73 ± 0.06	77.8‡
1.0	0.80 ± 0.03	0.57 ± 0.02	71.3‡	0.80 ± 0.03	0.59 ± 0.03	73.7‡
2.0	0.86 ± 0.05	0.61 ± 0.06	70.9‡	0.95 ± 0.05	0.58 ± 0.03	61.0‡
4.0	0.86 ± 0.05	0.61 ± 0.04	70.9‡	0.95 ± 0.05	0.60 ± 0.02	63.1‡

Mice were treated intravenously with peptidoglycan monomer in saline. Control groups received only saline. Results are means ± S.E. from two to four groups of mice; each group consisted of five mice.

* nmoles/mg protein.

† Significantly different from the control value, $P < 0.1$.

‡ Significantly different from the control value, $P < 0.05$.

Table 3. Effect of the disaccharide and pentapeptide moiety of peptidoglycan monomer on cytochrome P-450 content

Treatment	Time after administration (hr)	Cytochrome P-450 content (nmoles/mg protein)	Treated/control (×100)
Control	24	0.75 ± 0.01	—
	48	0.73 ± 0.03	—
Disaccharide	24	0.69 ± 0.02	92.0
	48	0.64 ± 0.02	87.7*
Pentapeptide	24	0.56 ± 0.03	74.7†
	48	0.57 ± 0.03	78.1†

Mice were treated intravenously with 0.5 mg (approx. 1 μmole) of each fragment. Results are means ± S.E. of 2–4 groups of animals; each group consisted of five mice.
* Significantly different from control values, $P < 0.1$.
† Significantly different from control values, $P < 0.05$.

monomer) (Table 1). On the basis of these findings, it could be expected that the other P-450 dependant monooxygenases and related drug metabolizing enzymes also might be affected. In *in vitro* experiments, enzyme activity was not affected.

In comparison to treatments with whole bacteria and cell wall fragments [6–9], peptidoglycan monomer elicited qualitatively similar although quantitatively different effects, the decrease in cytochrome P-450 content being less pronounced and the duration of the effect much shorter, for example the treatment of mice with *C. parvum* induced a significant decrease in cytochrome P-450 content detectable even after 14 days [6, 7]. It should be pointed out that our peptidoglycan monomer preparation did not contain endotoxin and was not pyrogenic and, therefore, the effects observed could be ascribed entirely to the peptidoglycan structure.

Concerning the possible mechanism of inhibition of the drug metabolizing enzyme system by an immunostimulatory compound, Farquhar *et al.* [9] suggested the connection between macrophage activation and related biological processes; especially by elaboration of the superoxide ion [22] triggered by an adjuvant and possible resulting damage of some components of the electron transport chain of liver enzymes. Soyka *et al.* [23] also discussed the role of the reticuloendothelial system in depression of drug metabolizing enzymes after administration of *C. parvum*. We have shown previously that the treatment with peptidoglycan monomer results in macrophage activation [15, 24] and the mechanism of action of synthetic immunostimulatory muramyl dipeptide also involves macrophage activation [25].

In summary, intra-venous administration of immunoadjuvant peptidoglycan monomer, GlcNAc-MurNAc-L-Ala-D-isoglutaminyl-*meso*-diaminopimelyl-D-Ala-D-Ala, originating from *Brevibacterium divaricatum*, to female mice resulted in a decrease of cytochrome P-450 content and 7-ethoxycoumarin-*O*-deethylase activity. The effect was time dependent and was most marked 24 and 48 hr after injection of peptidoglycan monomer. A significant decrease of cytochrome P-450 content was observed with doses ranging from 0.5 to 4.0 mg per mouse. A similar effect was induced by the peptide moiety of the peptidoglycan monomer, but not with the disaccharide.

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'Ruder Bosković' Institute
P.O. Box 1016
41001 Zagreb
Yugoslavia

ANDJA TREŠĆEC
SONJA ISKRIĆ
IVO HRŠAK
JELKA TOMAŠIĆ*

* Correspondence should be addressed to: Dr. Jelka Tomašić, Tracer Laboratory, 'Ruder Bošković' Institute, P.O. Box 1016, 41001 Zagreb, Yugoslavia.

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Formation of electrophilic chlorine from carbon tetrachloride—involvement of cytochrome P-450

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Despite many years of intensive research, the molecular processes which initiate carbon tetrachloride hepatotoxicity are not completely characterized. Although it is generally accepted that a reactive metabolite of carbon tetrachloride is responsible for its hepatotoxic effects [1–4], there are several possible reactive species which may be responsible for carbon tetrachloride toxicity. The chemically reactive compounds trichloromethyl radical [5, 6], dichloromethyl carbene [7, 8], and phosgene [9–11] are all products of carbon tetrachloride metabolism. We have shown recently than an electrophilic form of chlorine is a new product of aerobic carbon tetrachloride metabolism by rat liver microsomes [12, 13]. In the present report, we have shown that the formation of this unique metabolite is an enzymatic process catalyzed by cytochrome P-450.

Male Sprague–Dawley rats (180–250 g) were obtained from Taconic Farms, Germantown, NY. The animals were allowed free access to water and food (Purina Lab Rat Chow). Groups of three animals were treated daily with phenobarbital (80 mg/kg in saline) for 4 days prior to the experiment. Twenty-four hours after the last phenobarbital injection, liver microsomes were prepared as described elsewhere [11] and resuspended in 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.5). Protein content was determined by the method of Lowry *et al.* [14] with bovine serum albumin as a standard. The content of microsomal cytochrome P-450 was assayed by the method of Omura and Sato [15].

Unless otherwise indicated, incubation mixtures contained 4 mg of microsomal protein from phenobarbital-pretreated rats, 1.0 mM NADPH, 20 mM HEPES buffer (pH 7.5), 5 mM carbon tetrachloride, and 1 mM 2,6-dimethylphenol (DMP) in a total volume of 2 ml. The mixtures were incubated in sealed vials (20 ml) at 37° under an atmosphere of air. Electrophilic chlorine was quantitated by trapping the electrophilic chlorine with DMP to form 4-chloro-2,6-dimethylphenol (4-ClDMP). The amount of 4-ClDMP formed was measured by gas chromatography/electron ionization mass spectrometry as described previously [12, 13].

NADPH–cytochrome P-450 reductase was purified by the method of Yasukochi and Masters [16] to a specific activity of 12,000 units/mg protein. The reductase activity was assayed as described by Masters *et al.* [17]. The major form of cytochrome P-450 found in phenobarbital-pretreated male Sprague–Dawley rats was purified by the method of West *et al.* [18] to a specific content of 5.0 nmoles/mg protein. Although the values of specific activity or content of these purified proteins were lower than the reported maxima, sodium dodecyl sulfate (SDS) gel electrophoresis of the preparations by the method of Laemmli [19], as modified by Guengerich [20], revealed a single, apparently homogenous band. Loss of the protein prosthetic group during purification is the most likely cause of the low specific content [21, 22].

Cytochrome P-450 (2.0 nmoles) was reconstituted with dilaurylphosphatidyl choline (40 μg, suspended by sonication), NADPH–cytochrome P-450 reductase (10,000 units), DMP (1 mM) and carbon tetrachloride (5 mM) in 20 mM HEPES buffer (pH 7.5) in a final volume of 2 ml. The reaction was initiated by addition of NADPH (1 mM). After 60 min, the samples were analyzed for 4-ClDMP as described above.

Although the formation of electrophilic chlorine has been shown previously to require both oxygen and NADPH [12], the enzymatic nature of this process has not been fully studied. The rate of electrophilic chlorine formation, as measured by the trapping of electrophilic chlorine with DMP to form 4-ClDMP, was constant up to 30 min (data not shown). The correlation coefficient obtained after linear regression of six data points within this time period was 0.96. After incubation for 30 min under these conditions, only 20% of the cytochrome P-450 is lost. The rate of electrophilic chlorine formation was proportional to the protein concentration between 0.25 and 2.0 mg of microsomal protein/ml. The linear rates of electrophilic chlorine formation with respect to time and protein concentration demonstrate that enzyme cofactors were not rate limiting.

When the concentration of carbon tetrachloride in the incubation mixtures was varied from 0.25 to 10.0 mM (six data points), the rate of electrophilic chlorine formation reached a maximum asymptotically. The V_{max} and K_m, calculated by unweighted nonlinear least-squares regression [23], were 320 pmoles per 4 mg microsomal protein per 30 min and 0.98 mM respectively. Analysis of the data by means of a Lineweaver–Burk plot yielded similar values (V_{max} = 307 pmoles per 4 mg protein per 30 min, K_m = 0.88 mM, R^2 = 0.99). A maximum rate of 4-ClDMP formation was observed (Fig. 1) at a concentration of DMP of 0.5 mM although 1 mM yielded similar results. Higher concentrations of DMP (2.5 to 10 mM) decreased the rate of 4-ClDMP formation.

The role of cytochrome P-450 in the bioactivation of carbon tetrachloride to electrophilic chlorine was examined with inhibitors of cytochrome P-450 (Table 1) and with purified components of the mixed function oxidase system