Decrease in Hepatic CYP2C11 mRNA and Increase in Heme Oxygenase Activity after Intracerebroventricular Injection of Bacterial Endotoxin

Yoshinori SHIMAMOTO, Takafumi TASAKI, Hiroshi KITAMURA[1)], Kyogo HIROSE, Akio KAZUSAKA and Shoichi FUJITA*

Laboratory of Toxicology, Department of Environmental Veterinary Sciences and [1)]Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

(Received 11 November 1998/Accepted 2 February 1999)

ABSTRACT. We previously reported (Arch. Toxcol. 1998, 72, 492–498) that the differential decrease in the levels of hepatic cytochrome P450 (CYP) isozymes in rats was observed 24 hr after intracerebroventricular (icv) injection of bacterial lipopolysaccharide (LPS) at the dose ineffective (0.1 μg) when injected intraperitoneally (ip). Among CYP isozymes we examined, the male specific CYP isozyme, CYP2C11 was most severely affected by icv injection of LPS. In this study, we examined the gene expression of CYP2C11, the total P450 contents, the CYP2C11-dependent activity of imipramine N-demethylase (IMND) and protein of CYP2C11 10 hr after icv or ip injections of LPS. Intracerebroventricular injection of LPS significantly decreased the level of CYP2C11 mRNA (to 63% of saline icv control), the total P450 contents (to 70% of saline icv control), the IMND activity (to 74% of saline icv control), but not protein of CYP2C11 in rat liver. In contrast, ip injection of LPS at the same dose as icv did not significantly affect these parameters. Since CYP is a heme protein, we also measured the activity of heme oxygenase (HO) using the same rat liver microsomes. The HO activity was increased to 166% by icv injection of LPS and 135% by ip injection of LPS compared to corresponding saline control. It is suggested that icv injection of LPS down-regulates the expression of CYP2C11 at transcriptional level and that both the decrease in CYP2C11 mRNA and the increase in heme degradation may be involved in the decreased level of protein and activity of CYP2C11 by icv injection of LPS in rat liver.—KEY WORDS: CYP2C11, cytochrome P450, intracerebroventricular, lipopolysaccharide, messenger RNA.

J. Vet. Med. Sci. 61(6): 609–613, 1999

Hepatic cytochromes P450 (CYPs) are heme protein consisting of several isozymes that function as monooxygenases, which play a key role in the metabolism of clinically important drugs, chemical compounds and steroids, leading to detoxification or to activation of the parent compounds [15]. The alteration of drug metabolism under diseased conditions is of clinical importance. Many reports have shown that several hepatic CYP isozymes were down-regulated during systemic infection [5, 19] and inflammation caused by peripheral injection of lipopolysaccharide (LPS), bacterial endotoxin, [4, 11, 12] and inflammatory cytokines such as interleukin (IL)-1 [6, 13, 17], IL-6 [2, 13] and tumor necrosis factor (TNF)-α [1]. However, the reports indicating that central inflammation has any effect on the activities and levels of drug metabolizing enzymes are limited. We previously reported that central inflammation induced by intracerebroventricular (icv) injection of LPS at an ineffective dose when injected intraperitoneally (ip) had a differential inhibitory effect on the levels of proteins and activities of hepatic CYP isozymes in rat liver microsomes [16], suggesting that a central inflammation like meningitis differentially decreases the levels of hepatic CYP isozymes. Among hepatic CYP isozymes we examined, the male specific CYP isozyme, CYP2C11 was most severely affected 24 hr after icv injection of LPS. In this study, to clarify the mechanism of the down-regulation of this isozyme by icv injection of LPS, we first examined the expression of CYP2C11 mRNA and the level of protein of CYP2C11 and its drug metabolizing activity in rat liver 10 hr after icv injection of LPS. Furthermore, to estimate the effect of icv injection of LPS on heme degradation, we also measured the activity of heme oxygenase using the same rat liver microsomes.

* CORRESPONDENCE TO: Prof. FUJITA, S., Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, N18, W9, Kita-ku, Sapporo 060, Japan.

MATERIALS AND METHODS

Chemicals: LPS (*Escherichia coli* 0111: B4), imipramine hydrochloride and desipramine were purchased from Sigma (St. Louis, Mo.). 2-Hydroxyimipramine was kindly donated by Geigy (Basel, Switzerland). Hemin and bilirubin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of analytical grade.

Treatment of animals and preparation of liver microsomes: Male Wistar rats (Nihon SLC, Hamamatsu, Japan) weighing 210–230 g were housed in plastic cages at 24 ± 1˚C with a 12-hr light-dark cycle (lights on at 7:00–19:00) and given laboratory chow and water *ad lib*. All rats were anesthetized with sodium pentobarbital at a dose of 40 mg/kg by intraperitoneal (ip) injection and implanted with a sterilized polyethylene cannula (0.6 mm o.d.) in the right lateral cerebral ventricle (at the bregma, 3.6 mm deep, 1.5 mm lateral). They were used for the experiments after a 2-wk recovery period. LPS was dissolved in sterilized saline and was injected intracerebroventricularly (icv) or ip in

doses of 0.1 μg. The injection volume of icv or ip was 10 or 100 μl, respectively. Rats were anesthetized with CO_2 and then were killed by decapitation 10 hr after LPS or saline injection. A part of liver samples was obtained from decapitated rats and stored in liquid nitrogen. After perfusion of the liver with ice-cold 1.15% potassium chloride, the preparation of the hepatic microsomes and the measurement of cytochrome P450 contents were performed as previously described [14]. The protein concentration of each sample was determined by the method of Lowry *et al.* [9].

Microsomal enzymatic activities: The assays for the activities of imipramine N-demethylase (IMND), mainly catalyzed by CYP2C11, and 2-hydroxylase (IMOH), mainly catalyzed by CYP2D, were performed by a high performance liquid chromatographic method as previously reported [3]. This enzymatic reaction was conducted at 37°C for 1 min. The heme oxygenase activity was determined by measuring the formation of bilirubin [10, 24]. In brief, 2.0 m*l* of the reaction mixture containing 17 μM hemin as substrates and 2.0 mg/m*l* microsomal protein, 1.7 mg/m*l* rat liver cytosol, 2.0 mM $MgCl_2$, 0.85 mM G6P, 0.5 units/m*l* G6PDH, 0.8 mM NADPH and 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for 15 min and the reaction was stopped with 3.0 m*l* of ice cold chloroform. After centrifugation, the chloroform layer was spectrophotometrically measured with wavelength of 452 nm. Authentic bilirubin was used as the standard.

Immunoblot analysis: Liver microsomal proteins (15.6 μg) were separated by 10% sodium dodecysalphate (SDS) - polyacrylamide gel electrophoresis (PAGE) and were transferred to a nitrocellulose membrane [7, 20]. The protein of CYP2C11 was detected with specific polyclonal goat anti-rat CYP2C11 antibody (Daiichi Pure Chemicals, Tokyo, Japan). The bound second anti-goat antibody-horseradish peroxidase complex was visualized by staining with diaminobenzidine [22]. Spectral configurations of the immunoblotting were also analyzed using the methods of NIH imaging [8].

Northern blot analysis: Total RNA was extracted by the guanidine isothiocyanate method using ISOGEN solution (Nippon gene, Tokyo, Japan) according to the manufacturer's direction. Thirty micrograms of total RNA were denatured at 65°C for 15 min, separated on 1% agarose/formaldehyde gel, transferred to and fixed on a nylon membrane (Amersham, Buckinghamshire, UK). Complementary DNA (cDNA) probe for CYP2C11 was prepared by reverse transcription-polymerase chain reaction from total RNA extracted from rat liver. The CYP2C11 cDNA construct used as template was a 205 bp BamHI - EcoRI fragment encompassing bases 1580–1884 of the full-length cDNA [18]. The cDNA fragment was labelled with (α-^{32}P) dCTP using a multiprime DNA labelling kit (Takara, Japan). Radiolabelled cDNA was purified by Quick Spin Sephadex G-25 Columns (Boehringer Mannheim Corporation, Indianapolis, U.S.A.). Nylon membrane was hybridized with the labelled cDNA probes at 43°C for 12 hr, washed by 2x SSC - 0.1 % SDS at 43°C for 5 min × 2 and by 0.2x SSC -0.1 % SDS at 43°C for 5 min × 2 and was exposed to X-ray films for 1 day. The radioactivity was quantified using a BAS-1000 bioimage analyzer (Fuji Film, Tokyo, Japan). The membrane was also hybridized with a rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe as an internal standard.

Statistical analysis: All data are the means ± SEM. Statistical comparisons were made by analysis of variance, followed by Scheffe's *F* test. A p value less than 0.05 was regarded as statistically significant.

RESULTS

Total P450 contents and imipramine metabolism: We first examined the effect of icv or ip injection of LPS on the total P450 contents in rat liver microsomes at 10 hr after the treatment. Total P450 contents were significantly decreased to 70% of that in saline injected control rats by LPS injected icv, but not by ip (Fig. 1). Imipramine N-demethylase activity (IMND), mainly catalyzed by CYP2C11, was also decreased by icv-LPS to 74 % of that of icv-saline control (Fig. 2A). Imipramine 2-hydroxylase activity (IMOH), mainly catalyzed by CYP2D, was not changed by both LPS treatment (Fig. 2B). Intraperitoneal injection of LPS slightly decreased the activity of IMND to 85% of that of ip-saline-treated control rats, but the decrease was not statistically significant (p=0.23).

Immunoblot analysis: The slight decrease in protein of CYP2C11 was observed in only the group of rats treated with icv-injection of LPS. But the significant decrease in protein of CYP2C11 by icv-LPS was not observed (Fig. 3).

Northern blot analysis: Figure 4 shows a Northern blot of RNA isolated from rat liver 10 hr after the treatment with saline icv, LPS icv, saline ip and LPS ip. The intensity of the band observed in the liver of rats icv-treatment of

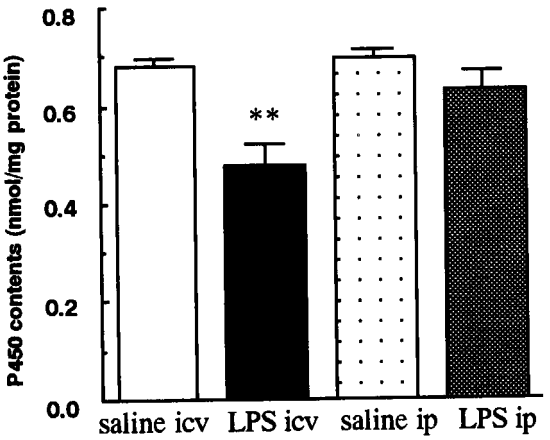

Fig. 1. The total P450 contents in rat liver microsomes 10 hr after icv or ip injections of LPS at a dose of 0.1 μg. Data are the means ± SEM of 4 rats for each treatment group. Key: (**) significantly different ($p<0.01$) from saline icv treatment.

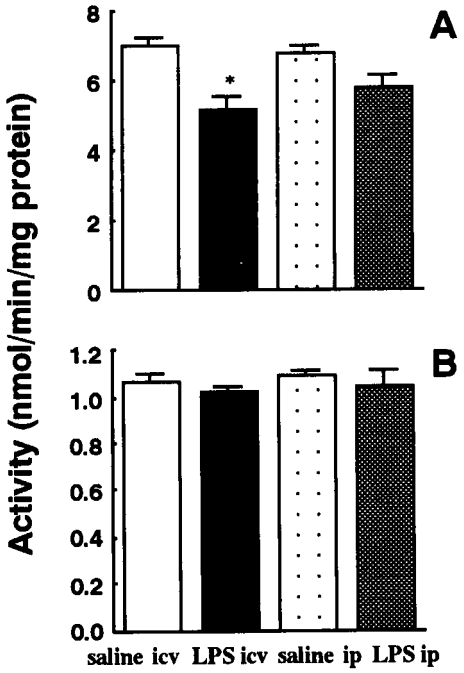

Fig. 2. A) The imipramine N-demethylase activity and B) imipramine 2-hydroxylase activity in rat liver microsomes 10 hr after icv or ip injection of LPS at a dose of 0.1 μg. Data are the means ± SEM of 4 rats for each treatment group. Key: (*) significantly different ($p<0.05$) from the saline icv treatment.

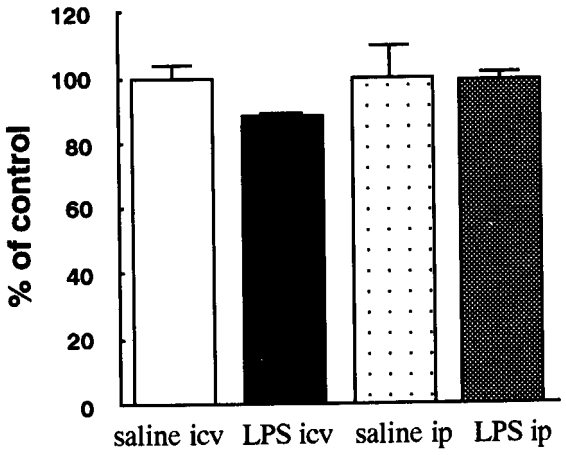

Fig. 3. Immunoblot of liver microsomes from rats 10 hr after treatments with LPS using antiserum to CYP2C11 isozyme. Lanes 1–4; hepatic microsomal proteins from rats treated with LPS icv at a dose of 0.1 μg (Lane 1), saline icv (Lane 2), LPS ip at a dose of 0.1 μg (Lane 3) and saline ip (Lane 4).

LPS was more decreased than that observed in rats received LPS ip. Bioimage analysis revealed that the level of CYP2C11 mRNA in the liver was significantly decreased to 63% of saline icv control by the icv treatment of LPS (Fig.

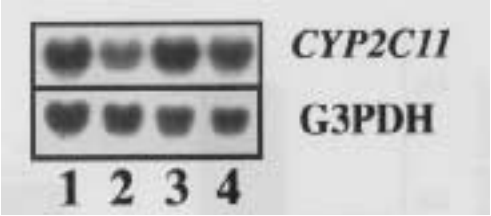

Fig. 4. Northern blots for CYP2C11 and G3PDH mRNA in rat liver 10 hr after treatments with LPS. Lanes 1–4; saline icv (Lane 1), LPS icv at a dose of 0.1 μg (Lane 2), saline ip (Lane 3) and LPS ip at a dose of 0.1 μg (Lane 4).

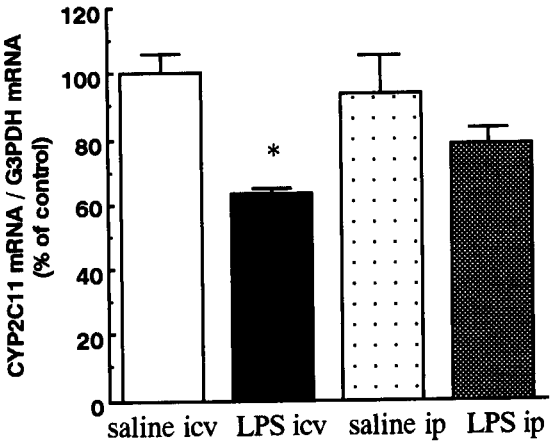

Fig. 5. Relative radioactivity in northern blots for CYP2C11 in liver from LPS-treated rats compared with controls. The radioactivity of the northern blots was analyzed by bioimage analyzer BAS 1000. For quantitative analysis, the CYP2C11 mRNA levels were normalized by G3PDH mRNA. Data are the means ± SEM of 4 rats for each treatment group and are expressed as a percentage of the mean value for saline icv treatment. Key: (*) significantly different ($p<0.05$) from each saline treatment.

5). Intraperitoneal injection of LPS tended to slightly decrease the levels of CYP2C11 mRNA to 83% of saline ip treatment, but statistical significance was not detected ($p=0.57$).

Heme oxygenase activity: The HO activity was increased by icv and ip LPS treatment to 166% and 135% of each saline treated group, respectively (Fig. 6). The extent of the increase in HO was greater in LPS icv- than in LPS ip-treated rats ($p<0.01$).

DISCUSSION

In this study, we found the decrease in the level of CYP2C11 mRNA at 10 hr after icv injection of LPS to rats. Although it is not clear whether the decrease in CYP2C11 mRNA is caused by the decrease in the rate of expression of CYP2C11 gene or by the acceleration of degradation of CYP2C11 mRNA, it is, at least, clear that this decrease is

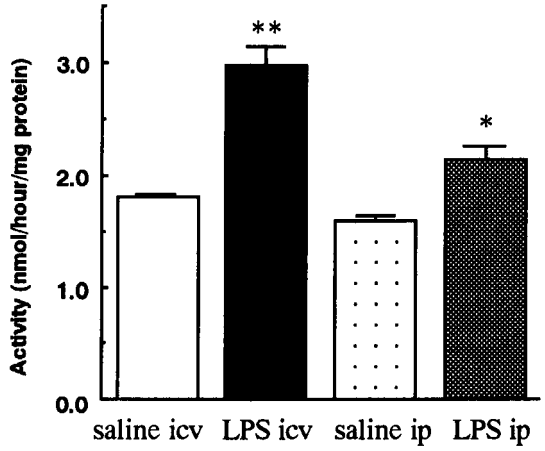

Fig. 6. The heme oxygenase activities in rat liver microsomes 10 hr after icv or ip injection of LPS at a dose of 0.1 μg. Data are the means ± SEM of 4 rats for each treatment group. Key: (*) and (**) significantly different ($p<0.05$) and ($p<0.01$), respectively, from the saline icv treatment.

apparently one of the cause of the reduced expression of CYP2C11. The HO activity was increased by icv injection of LPS coinciding with the decrease in the total P450 contents. This result is consistent with the results of Maines and Kanpas on the opposite correlation between the level of the oxidation of heme and the contents of the total P450 and microsomal heme in rat liver [10]. However, the difference of the extent of the decreases by icv-LPS between in the IMND (CYP2C11) activity and in the protein level of CYP2C11 was observed. Immunoblot analysis is able to detect not only holo- but also apo-CYP2C11 that is not containing heme. CYP protein without heme does not have an ability to catalyze the CYP dependent reaction. Therefore, it is suggested that the increase in the HO activity by icv-LPS is involved in the decrease in functional heme proteins including CYP isozymes, speculating that both the decrease in the level of CYP2C11 mRNA and the increase in heme degradation by icv-LPS contribute to the decrease in the hepatic microsomal contents of functional CYP2C11. In addition, the fact that CYP2C11 is more sensitive to icv-LPS than other CYP isozymes may suggest that protein turnover of CYP2C11 may be shortened under these condition. It appears that our results resemble the early reports on the decreased activities of hepatic CYP isozymes and the increase in the HO activity by peripheral inflammatory stimuli [21, 23]. On one hand, our study indicated that the IMOH activity, mainly catalyzed by CYP2D, was not significantly changed by icv-LPS after 10 hr as well as after 24 hr [16]. This might be explained by the speculation that the mRNA and/or protein of CYP2D isozyme may be stable or well protected that it is hardly affected by the single injection of the low dose of icv-LPS.

In summary, icv injection of LPS caused the decrease in the level of CYP2C11 mRNA and the increase in the level of the HO activity at 10 hr in rat liver. It is suggested that icv injection of LPS down-regulates the expression and the function of CYP2C11 at transcriptional as well as posttranscriptional level and that both the decrease in CYP2C11 mRNA and the increase in heme degradation may be involved in the decreased levels of activity and content of CYP2C11 24 hr after icv injection of LPS.

ACKNOWLEDGEMENT. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan to S.F. (No. 07558236), A.K. (No. 08456155) and H.I. (No. 09306021). This work was performed in partial fulfillment of requirements for the graduate Honors Program, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan.

REFERENCES

1. Bertini, R., Bianchi, M., Erroi, A., Villa, P. and Ghezzi, P. 1989. Dexamethasone modulation of *in vivo* effects of endotoxin, tumor necrosis factor, and interleukin-1 on liver cytochrome P-450, plasma fibrinogen, and serum ion. *J. Leukocyte Biol.* 46: 254–262.
2. Chen, Y. L., Florentin, I., Batt, A. M., Ferrari, L., Giroud, J. P. and Chauvelot-Moachon, L. 1992. Effects of interleukin-6 on cytochrome P-450 dependent mixed-function oxidases in the rat. *Biochem. Pharmacol.* 44: 137–148.
3. Chiba, M., Nishihara, E., Fujita, S. and Suzuki, T. 1985. Position selective sex difference in imipramine metabolism in rat liver microsomes. *Biochem. Pharmacol.* 34: 898–900.
4. Coto, J. A. and Williams, J. F. 1989. Effect of endotoxin to differentially affect cytochrome P-450 monooxygenase activities of untreated rats and animals induced with phenobarbital or 3-methylcholanthrene. *Int. J. Immunopharmacol.* 11: 623–628.
5. Kokwaro, G. O., Glazier, A. P., Ward, S. A., Breckenridge, A. M. and Edwards, G. 1993. Effect of malaria infection and endotoxin-induced fever on phenacetin O-deethylation by rat liver microsomes. *Biochem. Pharmacol.* 45: 1235–1241.
6. Kurokohchi, K., Yoneyama, H., Matsuo, Y., Nishioka, M. and Ichikawa, Y. 1992. Effects of interleukin 1a in the activities and gene expressions of the cytochrome P450IID subfamily. *Biochem. Pharmacol.* 44: 1669–1674.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227: 680–685.
8. Lennard, P. 1990. Image analysis for all. *Nature (Lond.)* 347: 103–104.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
10. Maines, M. D. and Kappas, A. 1975. Cobalt stimulation of heme degradation in the liver. Dissociation of microsomal oxidation of heme from cytochrome P-450. *J. Biol. Chem.* 250: 4171–4177.
11. Morgan, E. T. 1989. Supression of constitutive cytochrome P-450 gene expression in livers of rats undergoing an acute phase response to endotoxin. *Mol. Pharmacol.* 36: 699–707.
12. Morgan, E. T. 1993. Down-regulation of multiple cytochrome P450 gene products by inflammatory mediators *in vivo*: Independence from the hypothalamo-pituitary axis. *Biochem.*

Pharmacol. 45: 415–419.
13. Morgan, E. T., Thomas, K. B., Swanson, R., Vales, T., Hwang, J. and Wright, K. 1994. Selective suppression of cytochrome P-450 gene expression by interleukins 1 and 6 in rat liver. *Biochim. Biophys. Acta* 1219: 475–483.
14. Omura, T. and Sato, R. 1964. The carbon monoxide binding pigment of liver microsomes. *J. Biol. Chem.* 239: 2370–2378.
15. Porter, T. D. and Coon, M. J. 1991. Cytochrome P-450: multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J. Biol. Chem.* 266: 13469–13472.
16. Shimamoto, Y., Kitamura, H., Hoshi, H., Kazusaka, A., Funae, Y., Imaoka, S., Saito, M. and Fujita, S. 1998. Differential alterations in levels of hepatic microsomal cytochrome P450 isozymes following intracerebroventricular injection of bacterial lipopolysaccharide in rats. *Arch. Toxicol.* 72: 492–498.
17. Sindhu, R. K., Sakai, H., Okamoto, T. and Kikkawa, Y. 1996. Differential effect of interleukin-1a on rat hepatic cytochrome P450 monooxygenases. *Toxicology* 114: 37–46.
18. Strom, A., Mode, A., Zaphiropoulos, P., Nilsson, A. G., Morgan, E. and Gustafsson, J. A. 1988. Cloning and pretranslational hormonal regulation of testosterone 16 alpha-hydroxylase (P-45016 alpha) in male rat liver. *Acta Endocrinol.* 118: 314–320.
19. Topfer, F., Lenton, L. M., Bygrave, F. L. and Behm, C. A. 1995. Importance of T-cell-dependent inflammatory reactions in the decline of microsomal cytochrome P450 concentration in the livers of rats infected with Fasciola hepatica. *Int. J. Parasitol.* 25: 1259–1262.
20. Towbin, H., Staehlin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350–4354.
21. Williams, J. F. 1985. Induction of tolerance in mice and rats to the effect of endotoxin to decrease the hepatic microsomal mixed-function oxidase system. Evidence for a possible macrophage-derived factor in the endotoxin effect. *Int. J. Immunopharmacol.* 7: 501–509.
22. Winstone, S. E., Fuller, S. A. and Hurrell, J. G. R. 1992. Immunoblotting and immunodetection. pp. 10.18.11–10.18.16. *In*: Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. eds.), Greene Publishing and John Wiley, New York.
23. Yoshida, M., Egawa, K. and Kasai, N. 1982. Effect of endotoxin and its degradation products on hepatic mixed-function oxidase and heme enzyme systems in mice. *Toxicol. Lett.* 12: 185–190.
24. Yoshida, T. and Kikuchi, G. 1978. Reaction of the microsomal heme oxygenase with cobaltic protoporphyrin IX, and extremely poor substrate. *J. Biol. Chem.* 253: 8479–8482.