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Consequences of Challenge Infections with *Fasciola hepatica* on Rat Liver *P*450-dependent Metabolism of Sex Hormones

B. Biro-Sauveur, C. Eeckhoutte, J.-F. Sutra, C. Calléja, M. Alvinerie and P. Galtier*

Laboratoire de Pharmacologie-Toxicologie, INRA, 180 Chemin de Tournefeuille, 31931 Toulouse, France

The effect of single or repetitive fluke-infections on rat liver steroid hormone metabolism was studied. Fascioliasis was induced by oral administration of 20 metacercariae of *Fasciola hepatica* to rats, by week-6 (mono-infected) or 12 and 6 (bi-infected), or 12, 9 and 6 (tri-infected) before killing. Total microsomal cytochrome *P*450 and *P*450 isoenzymes were measured spectrophotometrically and by Western-blot analysis, respectively. Progesterone and testosterone metabolism were quantified by normal phase high performance liquid chromatography. In control rats, progesterone and testosterone were mainly converted to 2α- and 16α-hydroxymetabolites. In the liver of mono-infected rats, hepatic cytochrome *P*450 was significantly decreased by 36–64% whereas the expression of all investigated isoenzymes was decreased by 36–82% with the exception of the unchanged *P*4502E1. 16α- and 2α-hydroxylations of progesterone and testosterone were significantly decreased by 50–90%, these decreases were correlated with those of *P*4502B1/2 and *P*4502C11 isoenzymes, respectively. In bi- and tri-infected rats, steroid hormones were metabolized similarly to control rats. The return of steroid drug metabolizing enzyme activities to control level could be related to the immune response associated to the development of the animal resistance to the parasitic infection.

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INTRODUCTION

Liver fascioliasis is one of the most prevalent liver infections in humans and domestic ruminants. In animals, adverse effects of fascioliasis include both decreased meat and milk productions, decreased female fertility and increased veterinary cost. In previous studies, alterations of both oxidative and conjugative hepatic drug metabolizing enzymes have been reported in rats [1, 2] or lambs [3] experimentally infected with *Fasciola hepatica* metacercariae. The impairment of the oxidative drug metabolism is a result of the decrease in hepatic cytochrome *P*450 mediated activities. Altered drug metabolism is clinically important in that it may result either in therapeutic failure or in increased drug toxicity.

Cytochromes *P*450 represent a superfamily of enzymes that carry out mono-oxidation reactions [4, 5]. A small group of *P*450s contribute in pathways of steroidogenesis from cholesterol, including steps leading to the synthesis of mineralocorticoids, glucocorticoids, androgens and estrogens [6]. A large number of *P*450s that are expressed in liver are primarily responsible for oxidative degradation of xenobiotics or foreign compounds, such as drug, environmental pollutants and toxins. In addition, hepatic *P*450s exist as multiple molecular forms, some of which catalyze the stereo- or regioselective hydroxylations of steroids [7, 8]. Generally, it is believed that some *P*450-mediated metabolisms of endogenous steroids are pathways that are not physiologically relevant for steroid degradation but they occur due to the broad substrate specificities of *P*450s [5]. The activity of cytochrome *P*450 monooxygenases for steroid hydroxylation is dependent on age, sex, animal species and environmental factors [9, 10]. Progesterone and testosterone are known to be metabolized in liver, so it is important to investigate the

*Correspondence to P. Galtier.
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consequences of liver diseases on the hepatic metabolism of these hormones. Since natural fascioliasis is generally characterized by a repetitive infestation of breeding animals, the purpose of this work was to compare the effect of a single to multiple infections of rats by a similar parasitic dose of 20 metacercariae of *F. hepatica*. This study was also designed to discriminate between these effects on both the cytochrome *P*450 isoenzymatic pattern of rat liver and the hepatic metabolism of progesterone and testosterone.

MATERIALS AND METHODS

Chemicals

Progesterone, testosterone, androstenedione and 2α, 6β, 16α, 20α, 21-hydroxyderivatives of progesterone and testosterone were purchased from Sigma Chimie (la Verpillière, France). 6β and 7α-hydroxytestosterone were from Steraloids, Inc. (Wilton, U.S.A.). Anti-rat *P*450 2A1/2, 2C7, 2C11, 2E1, 3A1/2 and 2B1/2 were kindly provided by Dr F. J. Gonzalez (NIH, Bethesda, MD, U.S.A.) and Dr P. H. Beaune (INSERM U75, Paris, France), respectively. The reagents for gel electrophoresis and immunoblotting were supplied by Eurobio (Les Ulis, France). All other chemicals and solvents used were of the highest quality available and distilled deionized water was used in all studies.

Animals

Male Wistar rats (160–200 g) were obtained from Iffa-Credo (l'Arbresle, France). Rats were given free access to food and water during the experiments and housed three per cage. Animals were randomly divided into four groups of six rats. Fascioliasis was induced by oral administration of *F. hepatica* metacercariae suspended in a 1% Tween aqueous solution. The first group of rats (mono-infected) received 20 metacercariae of *F. hepatica* (Laboratoire d'Immunologie Parasitaire INRA, Nouzilly, France) 6 weeks before sacrifice. The second and third groups received respectively two and three doses of 20 metacercariae by weeks 12 and 6 (bi-infected) and 12, 9 and 6 (tri-infected) before sacrifice. Similar studies were carried out on an uninfected group.

Rats were killed simultaneously 6 weeks after the last dose corresponding to the end of parenchymal fascioliasis. Blood was withdrawn in heparinized tubes, centrifuged at 1500 *g* for 10 min and the plasma stored at −20°C until analysis.

Enzyme preparation

The liver was removed, freed of extrahepatic tissue, weighed, washed with an ice-cold saline solution, and blotted free of excess moisture. All subsequent operations were carried out at 0–4°C. Samples (8 g) were homogenized in 24 ml of ice-cold 0.1 M potassium phosphate buffer (pH 7.4; 0.1 M Tris–acetate; 0.1 M KCl, 1 mM EDTA; 0.02 mM butyled hydroxytoluene) in a glass–Teflon Potter homogenizer. The homogenate was centrifuged at 10,000 *g* for 30 min. The supernatant fraction was centrifuged for 1 h at 105,000 *g*, suspended in 0.1 M pyrophosphate HCl buffer (pH 7.5) and re-centrifuged for 30 min in order to obtain a cleaner microsomal fraction. The microsomal pellets were suspended in a solution consisting of 0.1 M EDTA, 20% glycerol potassium phosphate buffer (0.1 M, pH 7.4) and stored at −80°C until required. Protein was estimated according to Lowry *et al.* [11] with bovine serum albumin (BSA) as standard.

Enzyme assays

The activity of plasma aspartate aminotransferase (ASAT) was estimated at 37°C using the Biochemica test combination (Boehringer Mannheim, Ref. 125881, Meylan, France). The plasma concentration of α1 acute phase protein was determined by rocket immunoelectrophoretic determination [12]. Total microsomal *P*450 content was determined from the reduced carbon monoxide difference spectrum by the method of Omura and Sato [13]. *P*450 isoenyzmes 2A1/2, 2B1/2, 2C7, 2C11, 2E1 and 3A1/2 were separated by electrophoresis in 9% polyacrylamide gel in the presence of sodium deodecyl sulphate (SDS). The separated microsomal proteins (5 μg) were electrophoretically transferred to nitrocellulose membranes which were further incubated with anti-rat *P*450 produced in rabbit and finally with peroxidase-labelled anti-rabbit IgG. Relative content of each form of cytochrome *P*450 was estimated by densitometry of the Western-blots by using a Shimadzu CS 930 scanner; the band densities were compared between control and infected groups.

Measurement of microsomal progesterone and testosterone metabolism

The metabolic study on progesterone and testosterone was performed as follows: microsomal protein (1 mg) was pre-incubated under agitation at 37°C for 10 min with 5 μg steroid dissolved in ethanol and potassium phosphate buffer (0.05 M, pH 7.4) in a final volume of 2 ml. Following the addition of 50 μl NADPH (40 mM), incubations were carried out at 37°C for 10 min and stopped by the addition of 10 ml methylene chloride. These reactions showed a linear dependence on protein and substrate concentrations.

The samples were vigorously vortexed for 10 min, the organic phase removed by centrifugation for 10 min at 12,000 *g* and dried under nitrogen gas. The residues were dissolved in 100 μl of elution solvent and analysed immediately by HPLC [14]. The separation of progesterone and testosterone and their hydroxy-metabolites were achieved on a Partisil 5 mm packing column (Whatman S.A.). The chromatographic system consisted of a HPLC pump Kontron 422 and a Kontron 360 automatic injector. The flow rate of the mobile phase (hexane–ethanol, 93:7, v/v) was

maintained at 1 ml/min and the eluent was monitored at 240 nm using a Diode Array Detector 440 and a Data Station Kontron 450 integrator calculator. Retention times of the various compounds and metabolites were determined by injecting an ethanolic solution (10 μl) containing 100 ng of each authentic standard. The recovery of progesterone, testosterone and their metabolites was around 80%. Quantitation was achieved by integrating and relating the metabolite peak areas and comparing them to the areas of known amounts of pure metabolites standards chromatographed under the same conditions. The limit of quantification of the method was fixed at 0.005 nmol·min^{-1}·mg^{-1} of protein. The HPLC procedure permitted an efficient separation of progesterone and testosterone from their major metabolite during a one step chromatography assay of 40 min.

Analysis of data

Statistical significance was evaluated using analysis of variance including a complementary range test to compare the means. Correlations between *P*450 isoenzymatic contents and steroid hydroxylation activities were assayed. In all cases, a probability of $P < 0.05$ was considered significant.

RESULTS

Pathology and effect of fascioliasis on cytochrome P450 isoenzymes

No clinical signs were observed in any animal group during the experimental period. At autopsy, lesions characteristic of fascioliasis such as tortuous migration tunnels in liver parenchyma and bile duct fibrosis were present in mono-infected rats while a more atypical aspect characterized livers of bi-infected and especially tri-infected animals considered 6 weeks following the last infection. The numbers of flukes recovered were respectively 2.5, 4.2 and 4.8 adults/liver of mono-infected, bi-infected and tri-infected rats. By comparison of controls (74.5 U/ml), single and 2-fold infections with 20 metacercariae of *F. hepatica* provoked, respectively 138 and 38% increases in plasma ASAT activity while no change was observed in tri-infected rats. On the day of slaughter, α1 acute phase protein was only increased by 7.6-fold in the plasma of mono-infected rats but challenge infections did not provoke such an increase.

Changes in hepatic cytochromes *P*450 in *F. hepatica*-infected rats are shown in Table 1. In mono-infected rats, the statistical analysis clearly demonstrates the 36 or 64% decrease in total liver microsomal cytochrome *P*450 as expressed per mg of protein or per g of liver. The expression of all investigated isoenzymes was decreased by 36–82% with the exception of the unchanged *P*4502E1. These data are confirmed by Fig. 1 which illustrates the Western-blotting pattern of the various *P*450 isoenzymes assayed in the four groups of animals. The total cytochrome *P*450 and most of the measured isoenzymes recovered control values in the liver of bi-infected or tri-infected rats. However, *P*4502C7 and *P*4503A1/2 isoenzymes were respectively under- and over-expressed in the liver of these animals receiving a 3-fold infection of *F. hepatica*.

Hepatic metabolism of progesterone and testosterone

HPLC chromatograms of progesterone and testosterone and their metabolites after incubation with microsomes of control and multi-infected rats are displayed in Figs 2A and 2B. These peaks corresponding to progesterone and its mono-hydroxyforms: 2α, 6β, 16α (Fig. 2A) and to androstenedione, testosterone and its metabolites as 2α-OH, 6β-OH, 7α-OH and 16α-OH (Fig. 2B), respectively. In our experimental conditions, 14–15% of either progesterone or testosterone were unmetabolized in the presence of microsomal preparations from control rats, while 47% were transformed into various identified derivatives (Tables 2 and 3). In the case of progesterone, the major metabolites were 2α- and 16α-hydroxyprogesterone which represented each 21–22% of the parent compound whereas the

Table 1. Effects of single (mono-infected) and repetitive (bi-and tri-infected) fascioliasis on total microsomal cytochrome P450 and P450 isoenzyme levels

	Control	Mono-infected	Bi-infected	Tri-infected
Microsomal protein	15.00 ± 4.00[a]	8.35 ± 2.53[d]	11.00 ± 1.12[d]	11.70 ± 1.52
Cytochrome *P*450	1.00 ± 0.20[b]	0.62 ± 0.28[d]	1.00 ± 0.60	1.20 ± 0.17
Relative cytochrome *P*450 isoenzyme levels (%)	14.50 ± 3.00[c]	5.20 ± 2.35[d]	10.35 ± 2.40[d]	14.00 ± 2.00
*P*4502A1/2A2	100	52 ± 9[d]	97 ± 12	101 ± 13
*P*4502B1/2B2	100	21 ± 6[d]	91 ± 7[d]	107 ± 8
*P*4502C7	100	45 ± 12[d]	78 ± 6[d]	82 ± 9[d]
*P*4502C11	100	18 ± 4[d]	82 ± 9[d]	75 ± 19[d]
*P*4502E1	100	101 ± 12	93 ± 8	103 ± 46
*P*4503A1/3A2	100	66 ± 5[d]	114 ± 14[d]	112 ± 9[d]

Data are expressed as mean ± SD derived using 6 animals in each group.
[a]mg·g^{-1} of liver; [b]nmol·mg^{-1} of microsomal protein; [c]nmol·g^{-1} of liver; [d]significant difference from corresponding control values ($P < 0.05$).

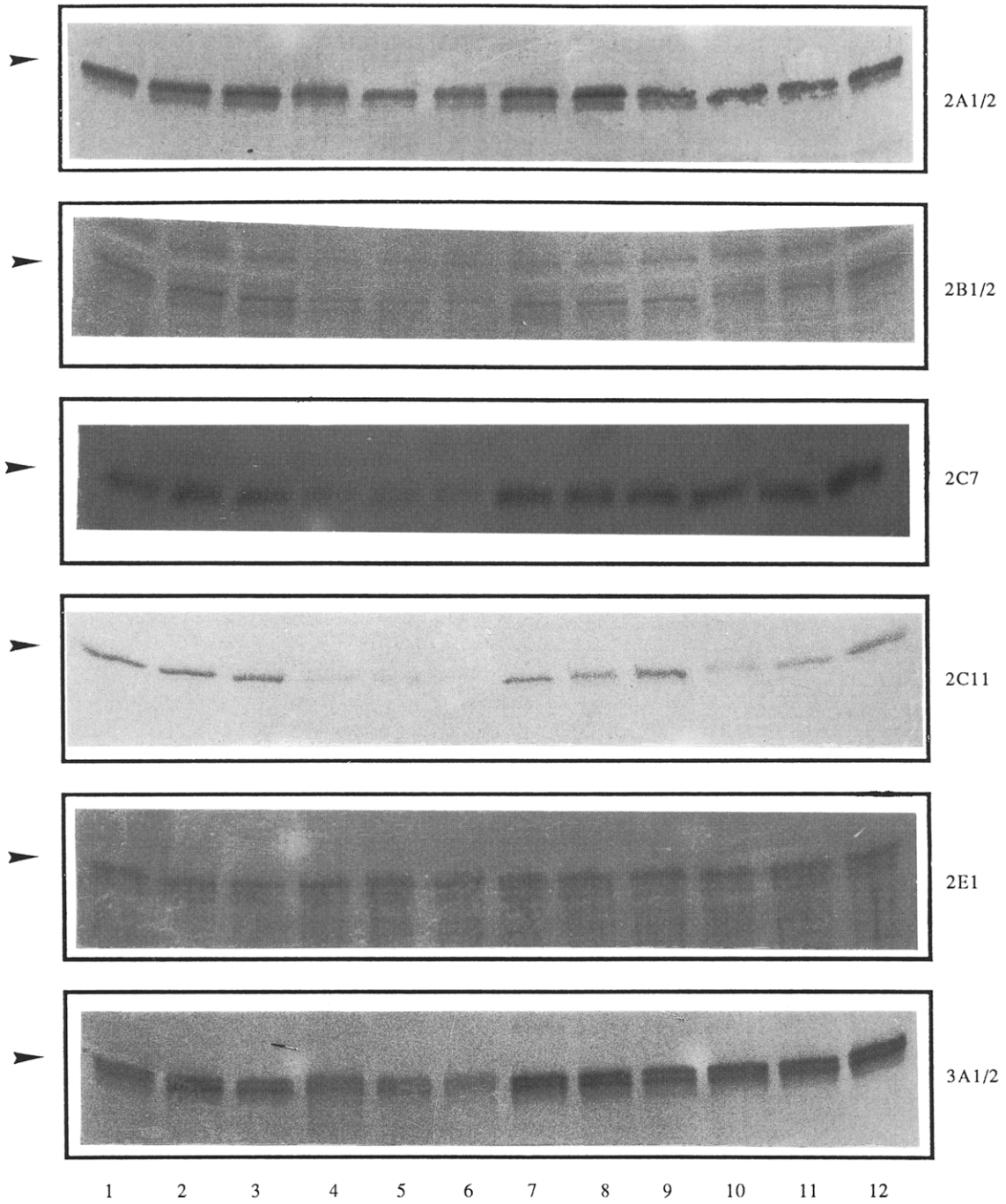

Fig. 1. Western-blot analysis of liver microsomes from control (1–3), mono-infected (4–6), bi-infected (7–9) and tri-infected (10–12) rats. Microsomal proteins (5 μg) were submitted to electrophoresis, transferred to nitrocellulose membranes and probed with antibodies prepared against rat liver cytochrome P450 2A1/2, 2B1/2, 2C7, 2C11, 2E1 and 3A1/2.

other hydroxylations corresponded only to 0.2–1.7%. Testosterone was converted mainly into its 16α-hydroxymetabolite (35%) and to a lesser extent (2.8–4.7%) into 2α- and 17-hydroxytestosterone or androstenedione (17-hydroxylation).

In mono-infected rats, unchanged progesterone and testosterone were increased 2.5 and 4.5 times, respectively. As indicated in Tables 2 and 3, 16α- and 2α-hydroxylations of both progesterone and testosterone were significantly decreased (50 to 90%) while the bioconversion of testosterone to androstenedione and 6β-hydroxylation of testosterone were lowered by 73 and 86%, respectively (Table 3). In bi- and tri-infected rats, steroid hormones were similarly metabolized by comparison to uninfected control rats. The only 6β-hydroxylation of progesterone appeared to be increased by 100 and 183%, respectively. However, its low intrinsic activity did not really influence the general metabolism of this hormone.

Correlations between cytochrome *P*450-isoenzy-

matic contents as determined by densitometry of Western-blots and the various steroid hydroxylations were assayed by taking into account all 24 individual paired data from uninfected and infected rats. Highly significant correlations were obtained between *P*4502C11 estimated levels and both 2α- and 16α-hydroxylations of progesterone or testosterone and androstenedione formation. There were also significant correlations between 16α-hydroxylation of hormones and cytochrome *P*4502B1/2 levels or between 6β-hydroxylation of progesterone and *P*450 3A1/2 contents.

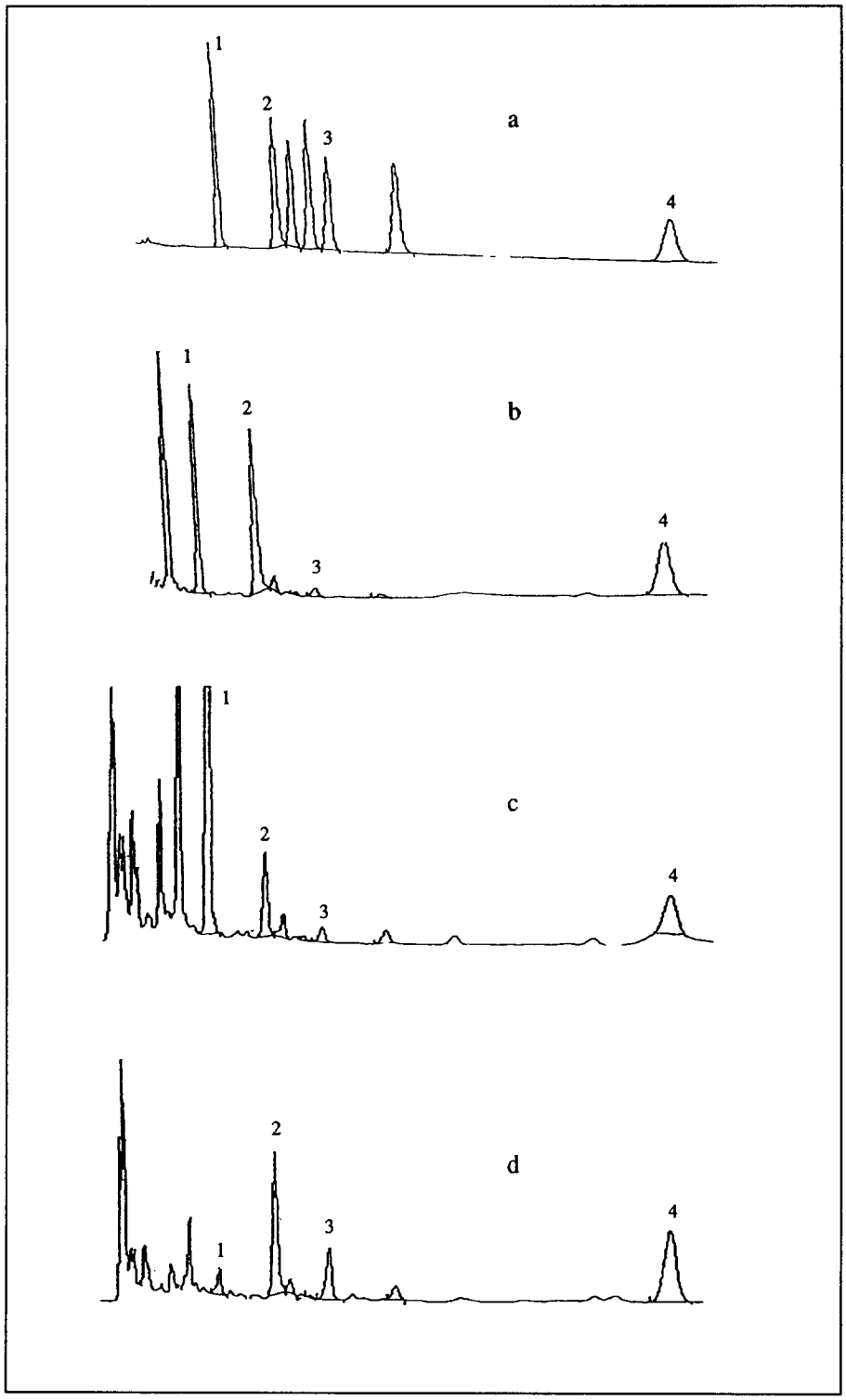

Fig. 2A. Progesterone hydroxylation: representative chromatograms (a) standard, (b) control rats, (c) mono-infected rats and (d) tri-infected rats. 1, progesterone; 2, 2α-OH; 3, 6β-OH; 4, 16α-OH.

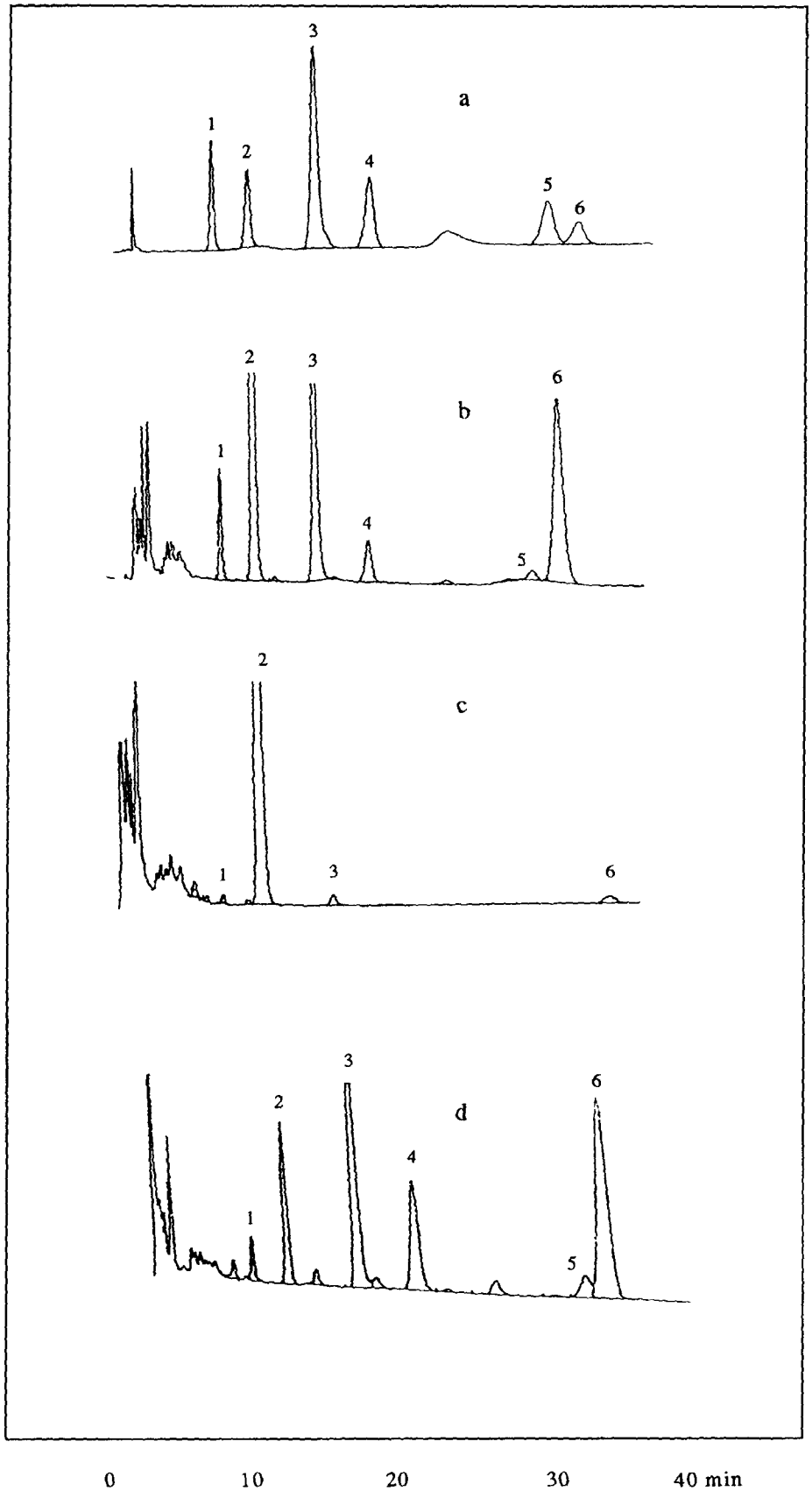

Fig. 2B. Testosterone hydroxylation: representative chromatograms (a) standard, (b) control rats, (c) mono-infected rats and (d) tri-infected rats. 1, androstenedione; 2, testosterone; 3, 2α-OH; 4, 6β-OH; 5, 7α-OH; 6, 16α-OH.

Table 2. Metabolic profiles of progesterone (Pg) in hepatic microsomes from control and F. hepatica-infected rats

	Control	Mono-infected	Bi-infected	Tri-infected
2 α-OH Pg[a]	0.34 ± 0.12[b]	0.15 ± 0.12[b]	0.32 ± 0.09	0.24 ± 0.03[b]
6 β-OH Pg	0.025 ± 0.007	0.022 ± 0.007	0.050 ± 0.017[b]	0.070 ± 0.024[b]
16 α-OH Pg	0.32 ± 0.13	0.16 ± 0.13[b]	0.33 ± 0.09	0.32 ± 0.02
20 α-OH Pg	0.014 ± 0.008	0.034 ± 0.011[b]	0.020 ± 0.008	0.040 ± 0.008[b]
21-OH Pg	0.008 ± 0.002	0.011 ± 0.008	0.017 ± 0.007	0.020 ± 0.007[b]

Data are expressed as mean ± SD derived using 6 animals in each group.
[a]nmol·min·$^{-1}$ mg^{-1} of microsomal protein; [b]significant difference from corresponding control values ($P < 0.05$).

DISCUSSION

The recovery of adult flukes and the pathological signs are proof of parasite infection and correspond to data reported previously [1, 2]. Liver of bi- and tri-infected rats were less affected, this could reflect a resistance to reinfection since it is well established that rats, like cattle, are susceptible to fluke infection and exhibit significant resistance associated with an immune response [15]. Unchanged values of plasma α1 acute phase protein in bi- or tri-infected animals on the day of slaughter could correspond to this resistance. The enhanced ASAT activity was observed in a previous study [1] and is consistent with liver cell inflammation and destructions caused by migration of juvenile flukes, particularly after the first and second infections while return to control activity in tri-infected rats confirms the atypical aspect of livers of this group of animals.

Because of the delayed response of liver to challenge infections, the decrease in total microsomal cytochrome *P*450 and *P*450 isoenzymes are essentially effective in mono- and bi-infected animals. From the decrease in microsomal protein, a transient increase in the fluid content of liver could partially explain the drop in *P*450 isoenzymes. However, the decrease in total *P*450, expressed per mg of microsomal protein, could be explained by a more specific process, such as liver inflammation occurring in mono-infected rats 6 weeks post-infection. In these animals, most *P*450 isoforms were found to be diminished with the exception of *P*4502E1. This result is in good agreement with our previous study describing the fall of *P*4502C11 (UT-A) in rats receiving a single dose of 25 metacercariae *F. hepatica* [2]. In tri-infected rats, cytochrome *P*450 content generally returned to control values and even appeared to be slightly increased, namely *P*4503A1/2 isoenzymes. Such a multi-phasic response could be related to the animal resistance to challenge infections and could also be associated with the dual effect of mediators of inflammation such as cytokines on cytochrome *P*450 expression in the course of primary or repetitive infection. By considering the elevation of α1 acute phase protein, an indicator of inflammation, liver injury would only be present in mono-infected rats. This increase could be consecutive with stimulation by interleukins. Interleukins 1, 6 and interferon γ inhibit cytochrome *P*450 expression [16–18], while interleukins 2 and 4 have been described to exert inducing properties towards certain of these hemoproteins [17, 19]. This hypothesis is based on the dual expression of these cytokines during either acute or chronic inflammatory processes and should be verified in further studies. Some reported interleukin 1 activities include induction of interleukin 2 receptor expression and cytokine gene expression [20], while biological activities ascribed to interleukin 4 include reduction in inflammatory processes through the down-regulation of interleukin 1, tumor necrosis factor and interleukin 6 production [21].

Our analytical method permits us to recognize six or five primary metabolites of either progesterone or testosterone, respectively. Hydroxylating activities of these hormones generally agree with previous studies using rat liver microsomes [22–25]. However 6β- and 16α-hydroxylations appeared to be respectively low and elevated by comparison to other studies. This

Table 3. Metabolic profiles of testosterone (T) in hepatic microsomes of control and F. hepatica infected rats

	Control	Mono-infected	Bi-infected	Tri-infected
Androstenedione[a]	0.045 ± 0.020	0.012 ± 0.005[b]	0.052 ± 0.007	0.030 ± 0.004[b]
2 α-OH T	0.080 ± 0.008	0.010 ± 0.008[b]	0.070 ± 0.010	0.086 ± 0.008[b]
6 β-OH T	0.045 ± 0.020	0.006 ± 0.002[b]	0.060 ± 0.020	0.060 ± 0.008[b]
7 α-OH T	0.025 ± 0.004	0.020 ± 0.004	0.020 ± 0.007	0.040 ± 0.004[b]
16 α-OH T	0.58 ± 0.08	0.06 ± 0.03[b]	0.50 ± 0.13	0.60 ± 0.09

Data are expressed as mean ± SD derived using 6 animals in each group.
[a]nmol·min·$^{-1}$ mg^{-1} of microsomal protein; [b]significant difference from corresponding control values ($P < 0.05$).

could be explained by the use of Wistar rats, Sprague–Dawley rats were used in most other studies. In an unpublished interstrain comparative assay, 6β-hydroxylation was found to be the predominant pathway of progesterone metabolism in this strain of rats by comparison to Wistar.

In mono-infected rats, the decrease in liver microsomal cytochromes *P*450 could explain the repressed activities of 2α- and 16α-hydroxylation of steroids and of androstenedione formation. These decreases could be related to those in *P*4502B1/2 and 2C11 isoenzymes which are well known to be especially involved in these metabolic pathways of progesterone [8] and testosterone [26]. This hypothesis is supported by the high significance of correlations between the levels of these isoenzymes and hydroxylating activities of steroids. The impairment of 6β-hydroxylation of testosterone could be explained by the correlated decrease in *P*4503A1/2 isoenzyme [26, 27]. It remains unclear why 6β-hydroxylation of progesterone was unchanged in liver of the same mono-infected rats. This oxidative activity is not very sensitive to inflammation developed in arthritic female rats receiving interleukin 1β [17]. In bi- and tri-infected rats, most of the steroid hydroxylases returned to control values or were even induced. These changes were correlated to those in cytochrome *P*450 2A, 2B and 2C isoenzymes. In tri-infected, only 6β-hydroxylation of progesterone was enhanced, correlating with an increased expression of cytochrome *P*4503A1/2. This result remains difficult to explain but it emphasizes the participation of the *P*4503A subfamily in 6β-hydroxylation of this steroid as it has been demonstrated in human or in sheep liver microsomes [14].

In conclusion, a single infection of rats by 20 metacercariae of *F. hepatica* leads to a decreased hepatic *P*450-dependent metabolism of both progesterone and testosterone. This result could explain the reduced ability to remove exogenous testosterone which was described in *F. hepatica*-infected post-puberal rams receiving testosterone intravenously [28]. This could also be related to the general observation of failure in maintaining pregnancy in fluke-infected farm animals as a possible consequence of reduced amounts of steroid hormones reaching target organs [29]. These changes are of a lesser extent in rats receiving a 2- or 3-fold parasitic dose. In these animals, the return to control steroid-metabolizing enzyme activities could be related to the immune response associated with the development of animal resistance to parasitism [15]. In this respect, the normal capacity of liver for handling drugs and endogenous compounds in the case of challenge infections, should have less consequences in the drug dosage adjustment or in retention of drugs or endogenous compounds in the animal's body by comparison to those in primary infected animals.

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