CHICKEN EGG YOLK AS AN EXCELLENT SOURCE OF HIGHLY SPECIFIC ANTIBODIES AGAINST CYTOCHROMES P450

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Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

Using chicken antibodies IgY (purified from egg yolks) against mammalian cytochromes P450 and by means of cytochrome P450 marker substrates, we found for the first time the presence of hepatopancreatic cytochrome P450 in crayfish *Orconectes limosus* (an inducible cytochrome P450 2B-like enzyme) and we were able to detect and quantify cytochrome P450 1A1 in microsomes of human livers. Expression levels of cytochrome P450 1A1 in human livers constituted less than 0.6% of the total hepatic cytochrome P450 complement. The results obtained in our study are clear examples that chicken IgY are suitable for cytochrome P450 detection and quantification. Due to the evolutionary distance, chicken IgY reacts with more epitopes on a mammalian antigen, which gives an amplification of the signal. Moreover, this approach offers many advantages over common mammalian antibody production since chicken egg is an abundant source of antibodies (about 100 mg IgY/yolk) and the egg collection is a non-invasive technique. In the case of antibodies against cytochrome P450 2B4, we documented fast and steady production of highly specific immunoglobulins. Thus, chicken antibodies should be considered as a good alternative to and/or superior substitute for conventional polyclonal antibody produced in mammals.

Keywords: Yolk antibody; Chicken egg; IgY application; Immunoassays; Cytochrome P450; Crayfish; Human liver; Antibodies; Antigens; Immunoglobulins.

Antibodies are widely used in clinical practice for determination of either own body antibodies (e.g. HIV test, IgE level), proteins associated with various diseases (cancer markers) or low-molecular-weight compounds (e.g. progesterone). Techniques such as immunodiffusion and immunoelectro-

phoresis, ELISA and RIA[+], or Western blotting are indispensable tools for determination or detection of various proteins (antigens). Also inhibitory antibodies, blocking a ligand–receptor interaction or affecting enzyme activity, are widely exploited in protein biochemistry. Outside the field of diagnostics, antibodies are applied as "antidotes" in neutralization of toxins (tetanus toxin, snake venom)[1] or as means of passive immunization against e.g. microbial or viral infection (diarrhoea caused by *Escherichia coli* or rotavirus)[2,3].

Although the use of monoclonal antibodies has increased in the last decades, for many purposes the polyclonal antibodies, obtained by hyperimmunization of experimental animals, are still sufficient, if not superior. In the most usual case, the antibodies are obtained from blood of experimental animals (e.g. mouse, rabbit, goat, pig, horse) collected either by repeated bleeding or heart puncture resulting in death of the animal. Another source of antibodies is mammalian colostrum. Furthermore, evidence is accumulating that immunoglobulins obtained from avian eggs (egg antibodies) possess properties comparable or in some regards even better than those of mammalian ones[4,5]. Despite this fact, the use of avian antibodies is still much less common, perhaps due to some differences in properties of avian immunoglobulins, which bring a need to modify or check the usual protocols for immunological techniques based on mammalian antibodies. As the preparation and use of avian antibodies is not well known yet, we would like to introduce this promising and still a relatively new tool in more detail.

Birds protect their offsprings by passive immunization using immunoglobulins present in their eggs. During egg fertilization, blood immunoglobulins (corresponding to mammalian IgG) are concentrated in the yolk, while IgA and IgM are secreted into the egg white[6]. The concentration of IgG in egg yolk (10–25 mg/ml) is 1.3–1.9 times higher compared with that

+ *Abbreviations used*: BCA, bicinchoninic acid; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitrobluetetrazolium; BSA, bovine serum albumin; BR, benzylresorufin; CO, carbon monooxide; CYP, cytochrome P450; ELISA, enzyme-linked immunosorbent assay; ER, 7-ethoxyresorufin; EC, ethoxycoumarin; FITC, fluorescein isothiocyanate; IgY, egg yolk immunoglobulin; Mr, relative molecular weight; PB, soluble phenobarbital; PBS, phosphate buffered saline solution; PBSM, 5% skim milk solution in PBS containing 0.3% Triton X100; PBST, PBS containing 0.3% Triton X100; PEG, poly(ethylene glycol); p*I*, isoelectric point; RIA, radioimmunoassay; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

in hen blood[7]. The yolk sack serves as a nutrition source of newly-hatched chicken as well as means of their passive immunization.

In contrast to mammals, birds possess only three classes of immunoglobulins: IgA, IgG and IgM. Moreover, the structures of the corresponding immunoglobulins of both animal classes are significantly different, namely within the heavy chains. While avian IgG, like mammalian IgE and IgM, comprises four constant domains, mammalian IgG contains only three. Most likely in the case of mammals, one constant domain was reduced to a short segment, forming the hinge region typical of mammalian IgG[8]. Because of differences between mammalian and avian IgG, Leslie and Clem[9] introduced the term IgY (standing for egg yolk) for avian IgG-like class of immunoglobulins.

IgY (in contrast to IgG) does not react with rheumatoid factor, mammalian Fc receptors and does not activate the mammalian complement system. These properties of chicken antibodies make them a superior tool for immunodetection techniques with mammalian sera as samples[10]. In addition, IgY shows a very low affinity for binding of protein A and G[11], explaining why sorbents with immobilized protein A and G are not applicable to antibody purification as it is common for IgG.

Since the heavy chain (H_C) of IgY is one constant domain longer than that of IgG, its relative molecular weight increases to 64 000 as determined by SDS–polyacrylamide gel electrophoresis. Molecular weight of the light chain (L_C) is around 28 000[12]. The absence of the hinge region in IgY reduces motion of the arms with variable domains resulting in a steric hindrance which affects the crosslinking of a binary complex antigen–antibody necessary for efficient precipitate formation[13]. On the other hand, there is an accumulating evidence in the literature showing chicken IgY to be also precipitating antibodies[14].

For more than a century, the chicken egg has been well known as a rich source of immunoglobulins[15]. An average egg yolk contains about 100 mg IgY. Although the immunoglobulin concentration in yolk (10–25 mg/ml)[7] is lower than in mammalian serum (e.g. for rabbit – 35 mg IgG/ml)[12], the every day production of eggs overcomes this disadvantage. When rabbits and chicken are compared in terms of antibody production, the somewhat surprising conclusion is that one chicken produces in a year about 25 g of IgY, obtainable from blood of 30 rabbits within the same period of time[12]. The specific antibodies against the antigen used for immunization comprise 0.1–10% of total IgY produced, depending on the antigen[16].

Thanks to the evolutionary distance between birds and mammals, the chicken is superior for the production of antibodies against conserved

mammalian antigens, which are hardly immunogenic for mammals[17]. Chicken IgY is usually produced against a greater number of antigenic epitopes on a mammalian antigen compared with mammalian IgG. This leads to an amplified signal and greater sensitivity in immunochemical methods. Another advantage of IgY lies in the possibility of developing high titre chicken antibodies even when low doses of mammalian antigen (0.001–0.01 mg/dose) are applied[18]. Thus, for preparation of antibodies against conserved mammalian antigens, chicken IgY technology should be chosen. This makes the IgY technology particularly interesting also in the field of research of cytochrome P450 (CYP), the enzyme playing a major role in the metabolism of drugs and activation of carcinogens, where many similar enzyme forms have to be studied. Recently, the hen egg yolk antibodies against CYP101 (P450cam) were successfully prepared and used for epitope mapping of this enzyme[19].

The relatively inexpensive production of large quantities of IgYs predestines them for prophylaxis and/or acute passive immunization[2,3,20–23]. Common mammalian antibodies were used for these purposes only exceptionally to treat cases of emergency, because of their high cost. Another, rather new medical area of application of IgYs is xenotransplantation[24], where they block human antiporcine xenoantibody binding that is expected to inhibit xenograft rejection by endogenic antibodies. The advantage of IgYs in this respect is that they do not activate the human complement system[5].

Another promising application of IgYs consists in human hemoclassification[25]. IgYs, possessing a lower p*I* value than mammalian IgGs, are applicable to rocket electrophoresis to quantify immunoglobulins of mammalian sera[26] without need of carbamoylation to differentiate values of isoelectric point as it is common for mammalian IgGs. Moreover, conjugates of IgY with horseradish peroxidase, FITC or biotin, can be used for common immunochemical procedures[18].

Immobilized IgY for immunoaffinity chromatography of various compounds described in several publications is another perspective way of IgY application[27]. Bound antigen is usually eluted in high yields (97%) under milder conditions (pH 4) than from columns based on IgG, hence this process is suitable for purification of e.g. acid-labile antigens.

The major limitation preventing a wide application of IgYs from egg yolks lies most probably in their purification. Whereas the mammalian antibodies are easily isolated from blood as antisera, IgY comprises only about 5% of egg yolk proteins dispersed in yolk lipid emulsion together with lipoproteins and glycoproteins[28]. Various procedures were developed for IgY

purification[29,30]. The first step always consists in removal of the lipid fraction (extraction into organic solvent, precipitation, freezing or hydrophobic chromatography). IgY is then usually prepared in the second step from the water-soluble protein fraction by fraction precipitation or chromatography on ion-exchange, thiophilic or size-exclusion columns[31–33]. In most protocols, however, three purification steps are needed to obtain a high-purity (98%) final preparation, with the yield of 70–100 mg IgY per one egg. To prepare monospecific antibodies, affinity chromatography on immobilized antigen is usually used. Specifically bound IgY is eluted with strong acidic or alkaline buffer[5].

Purified IgYs with preserving agent (e.g. sodium azide) show high stability when they are stored at 4 °C. They retain their activity for more than 10 years[34]. Thus, one might expect that application of avian egg antibodies, namely those of chicken (*Gallus domesticus*), will gain wide acceptance as an appropriate substitute for and/or superior alternative to mammalian ones.

The aim of the present study was the preparation of chicken antibodies against integral membrane proteins, cytochromes P450, and utilizing them to resolve two problems, which have not been clearly solved until the present time. The first of them was to find whether cytochrome P450 enzymes are expressed in a freshwater crayfish, *Orconectes limosus*. Chicken polyclonal antibody raised against CYP2B4 was utilized in this study. The second goal of our study was to explain still conflicting evidence for the expression or inducibility of CYP1A1 protein in human livers[35–37]. Chicken anti-rat CYP1A1 was employed in this case.

EXPERIMENTAL

Chemicals

7-Hydroxy-3*H*-phenoxazin-3-one (resorufin), its ethyl (ER), pentyl (PR), benzyl (BR) ethers, 7-ethoxycoumarin (EC), umbelliferone, poly(ethylene glycol) 6000 (PEG), glucose 6-phosphate, anti-chicken IgY rabbit IgG-alkaline phosphatase conjugate, and BCIP/NBT-blue tablet substrate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 4-nitrophenyl phosphate, glucose 6-phosphate dehydrogenase from Serva (Heidelberg, Germany); phenobarbital (PB) from Farmakon (Olomouc, Czech Republic); Emulgen 911 from Atlas Co. (Japan); bicinchoninic acid from Pierce (Rockford, IL, U.S.A.); CNBr-activated Sepharose 4B from Pharmacia (Uppsala, Sweden) and the proteinase inhibitor Complete™ from Boehringer (Mannheim, Germany). All other chemicals were of reagent grade or better purity and were purchased from Lachema (Brno, Czech Republic).

Crayfish Microsomal Preparations

Freshwater crayfish, *Orconectes limosus* "RAFFINESQUE" (8 males and 8 females) caught in the brook Pšovka (near Mělník, Czech Republic) were divided into two groups with the same size and sex composition. Animals were kept in 15-liter containers and fed with carp meat during the experiment for six days before being sacrificed. The water environment was continuously aerated. Control animals were kept in plain water (5–9 °C). The treated animals were exposed for 7 days to 0.1% solution of phenobarbital at 5–9 °C. After sacrifice, the hepatopancreases were collected in liquid nitrogen, and the microsomal fraction was prepared by a difference centrifugation using a modification of the method of Lindström-Seppä[38]. All buffers contained the mixed proteinase inhibitor Complete™.

Human Microsomal Preparations

Microsomes from livers of eight human donors who died in traffic accidents (a gift of B. Szotáková, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic) were isolated as described[39]. The age of donors (5 men, 3 women) ranged from 24 to 70 years. All the donors had unknown drug history and none had a history of alcohol abuse. Supersomes™, microsomes isolated from insect cells transfected with *Baculovirus* constructs containing cDNA of one of the following human cytochromes P450 (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) and co-expressing NADPH:CYP reductase, were purchased from Gentest Corp. (Woburn, MA, U.S.A.).

Analytical Methods

The concentration of cytochrome P450 was determined from difference spectra of reduced CO complex[40], total concentration of protein by the bicinchoninic acid assay (BCA) using bovine serum albumin (BSA) as a standard[41]. Cytochrome P450 dealkylation (dearylation) activities were measured fluorimetrically using EC, ER, PR, and BR as substrates[42].

Chicken Antibody Preparation and ELISA

Antibodies were prepared from egg yolks of immunized hens. Leghorn chickens (≈1 year old) were kept in cages (one animal per cage). Chickens were immunized by three subcutaneous injections (into wings) in one-week intervals of CYP antigens (0.1 mg/animal). Antigens, rat recombinant CYP1A1 and rabbit CYP2B4 were prepared from *E. coli*, infected with CYP1A1 expression vector[43], and liver of phenobarbital-treated rabbit[44], respectively. As judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), both CYPs were highly purified protein preparations. CYP antigens were emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for subsequent boosters. An injection volume was 0.5 ml of emulsion per animal. The immunoglobulin fraction (crude antibody preparation) was isolated from pooled egg yolks as described by Polson et al.[14,45]. Dilute yolks (1:2) in PBS were precipitated with poly(ethylene glycol) 6000 (PEG) to final concentration of 3.5%. IgY were then precipitated from the resulting supernatant using 12% PEG.

The specific antibody content was tested by ELISA. Antigen solution (4 μg/ml in 50 mM sodium carbonate buffer, pH 9.6) at 100 μl per well was used to coat an ELISA plate (Nunc–Polysorp, Denmark) and incubated at 4 °C overnight. After washing three times with

PBS (PBS consists of 3 mM sodium phosphate in 135 mM sodium chloride, pH 7.2) containing 0.1% Tween 20 (PBST), each well was loaded with 150 μl of 2% solution of ovalbumin in PBST and incubated at 37 °C for 1 h. Wells were washed three times with PBST and then in doublets loaded with 100 μl of antibody solution in PBS (preimmune and after immunization, concentration series 50, 25, 12, 6, 3, 1.5 μg/ml). After washing three times with PBST, to each well 100 μl of dilute alkaline phosphatase-conjugated rabbit anti-chicken IgG in PBS was added (2000 times diluted commercial preparation) and incubated at 37 °C for 1 h. After washing with PBST, 100 μl of substrate solution (1 g/l 4-nitrophenyl phosphate in carbonate buffer) was added. After 10 min, reaction was stopped by addition of 50 μl of 3 M NaOH to each well and the color developed was assayed at 405 nm with an ELISA reader ELX 800 (Bio-Tek Instruments, Winooski, VT, U.S.A.). Fractions containing the highest content of specific antibodies were pooled and further purified by affinity chromatography to delete balast proteins without lost of antibody biological activities. The affinity chromatography was carried out as follows.

Protein fraction (24 mg) precipitated with poly(ethylene glycol) 6000 (8–12%) of Emulgen 911-solubilized microsomes[44] prepared from liver of phenobarbital-treated rabbit were immobilized on CNBr-activated Sepharose 4B (4 ml) according to the protocol recommended by manufacturer. The resulting affinity sorbent (rich in CYP2B4) was incubated overnight (end-over-end mixing) in two separate batches (2 ml each) with 40 mg of crude antibody preparation (against CYP1A1 or CYP2B4) at 4 °C. Antibodies against CYP1A1 were collected from the first batch from unretained fraction. Anti-CYP2B4 immunoglobulins were eluted from the second batch. The column was first washed with PBS (1 M NaCl), and then eluted with 0.2 M glycine buffer, pH 2.5, followed by an immediate neutralization with 1 M Tris-HCl, pH 8.0. Immunoglobuline fractions were dialyzed against 50 mM ammonium hydrogen carbonate solution and lyophilized. Partially purified immunoglobulins reacted with respective antigens with efficiency analogous to that of crude immunoglobulin fractions.

Western Blot Analysis

Detection of human CYP1A1, 1A2, and putative crayfish cytochrome P450 with respective anti-cytochrome P450 chicken antibodies was carried out on Western blots[46] of human and crayfish microsomes, respectively. Human microsomal CYP1A1 and 1A2 proteins were probed with a chicken polyclonal antibody raised against rat recombinant CYP1A1[47]. This antibody recognized both CYP1A1 and 1A2 in rat liver microsomes as well as human CYP1A1 and 1A2 expressed in Supersomes™ as two distinct bands. On the other hand, it did not cross-react with any of all other available human recombinant CYPs (1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4). Chicken polyclonal antibody raised against rabbit CYP2B4 recognized CYP2B4 purified as described elsewhere[44] as one band.

Standards (CYP1A1 and CYP1A2 expressed in Supersomes™, 0.1–1.5 pmol/well) and human hepatic microsomes (75 μg/well) or crayfish (50 μg/well) and rabbit (5 μg/well) microsomal proteins were electrophoretically separated on SDS-PAGE[48] using 8.5 or 10% separation gel and then electro-transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.) on a semi-dry blot apparatus (Biometra, Germany) at 0.8 mA/cm^2 for 10 min and 2.0 mA/cm^2 for 45 min[46]. The membrane was then incubated at 4 °C overnight with a 5% skim milk solution in PBS containing 0.3% Triton X100 (PBSM) to block unoccupied membrane binding sites. Afterwards, the membrane was cut into two parts. The first was incubated for 2 h under shaking with the preim-

mune antibody, the other with the specific antibody. For detection of CYPs in crayfish and human hepatic microsomes, chicken antibodies were diluted to 1 and 10 μg/ml of PBSM, respectively. After washing, the parts of the membrane were individually incubated (1 h) with the secondary antibody (rabbit anti-chicken IgY-alkaline phosphatase conjugate, commercial preparation diluted 2000 times with PBSM). After washing with PBSM and PBS, the membranes were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitrobluetetrazolium (BCIP/NBT) substrate as recommended by the manufacturer's protocol. The resulting membranes were scanned and the CYP content in developed zones estimated using Elfoman software (Ing. Semecký, Czech Republic) based on CYP standards of known concentration.

The bands corresponding to CYP1A1 protein of two human hepatic microsomal samples (samples H5 and H6, see Fig. 3) were excised from a PVDF membrane and subjected to N-terminal sequencing, on a Protein Sequencer LF3600D (Beckman Instruments) according to the manufacturer's manual.

RESULTS AND DISCUSSION

Since cytochromes P450 of our interest are frequently of mammalian origin, chicken as an evolutionarily distant animal was used to produce anti-CYP antibodies. In the present paper, we focus on two examples of successful application of chicken antibodies for detection/quantification of CYPs in human and crayfish microsomal samples. The prepared IgYs were used as primary antibodies in ELISA and Western blotting and also as secondary antibodies when conjugated with peroxidase.

In response to antigen (CYP protein) injection, the chicken continuously produces high amounts of specific antibodies. Figure 1 shows a typical time

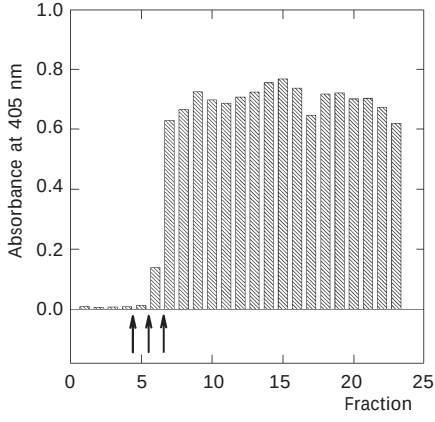

FIG. 1

Production of IgY against CYP2B4 determined by ELISA and expressed as the increase in absorbance at 405 nm. Fractions were prepared from pooled yolks of eggs hatched within a week period. Arrows mark antigen injections. For the assay, total IgY concentration in fractions was adjusted to 15 μg per ml PBS

course of specific antibody production against CYP2B4. Starting already from the fifth week after the first antigen injection, the maximal antibody production is usually reached and kept for at least 6 months. A time-dependence of antibody production against rat CYP1A1 was similar to that found for anti-CYP2B4 antibodies (results not shown).

Immunodetection of Crayfish Cytochrome P450

The inducibility of some CYP isoforms (the concentration rise after contact of the individual with inducer chemicals) provides a specific "chemical memory", ideal for biomonitoring. A drawback of the vertebrates, e.g. fish, studied in many previous works, is that these species usually inhabit a fairly large area, which makes the exact localization of the pollution difficult. For this reason, many invertebrates may be more suitable. The potential use of one of them, a freshwater crayfish, *Orconectes limosus*, for such purposes was evaluated in our laboratory. Cytochromes P450 in this crayfish species have not been detected as yet. Therefore, in the present study, we attempted to determine the presence of these enzymes in hepatopancreas of this species. Furthermore, we tried to find whether the cytochromes P450 in this freshwater crayfish might be induced with phenobarbital, which is known to be a potent inducer in vertebrates. Because no crayfish cytochrome P450 protein was available as antigen, rabbit CYP2B4, the enzyme inducible with phenobarbital, was employed. First, detection of CYP enzymes in hepatopancreatic microsomes by the Western blot technique using an affinity-purified chicken antibody against rabbit CYP2B4 was attempted. In hepatopancreatic microsomes this antibody cross-reacted with a single protein band of a relative molecular weight of 50 000, which is similar to that of rabbit CYP2B4 (Fig. 2). Closely related values (50 000–52 000) are reported for other crustacean CYPs [49]. Thus, the specifically detected protein is most likely a microsomal CYP of the crayfish *Orconectes limosus*.

Exposure of crayfish to phenobarbital in their environment caused no significant increase (<10%) in the cytochrome P450 specific content (fraction of CYP of the total microsomal protein content) in comparison with a control group of animals kept in tub water. However, microsomal metabolic activities of marker substrates (PR, BR) of a mammalian CYP2B subfamily were enhanced 3–4 times relative to the control (Table I). Comparison of microsomal samples from PB-exposed and untreated animals by Western blot analysis, shown in Fig. 2, did not reveal marked differences in the intensity of developed crayfish protein bands. Since the cytochrome P450 specific content was not much changed, it is highly probable that PB

induced a single CYP isoform, while the other(s) proportionally decreased as is common case for mammalian CYPs. Moreover, anti-CYP2B4 antibody may cross-react with several immunologically related crayfish CYPs of an almost identical molecular weight. This, together with the activity found with resorufin derivatives, suggests that the crayfish contains an inducible CYP2B-like protein of molecular weight about 50 000.

TABLE I
Effect of phenobarbital treatment on CYP induction in crayfish hepatopancreatic microsomal samples

Sample	CYP specific content[a]	Metabolic activity[b]		
		BR	PR	ER
Untreated	0.23	492	54	90
PB-treated	0.25	2010	222	144

[a] The specific content was expressed as the amount of CYP (nmol, assayed as CO adduct) per mg of total protein (BCA assay). [b] In pmol/min/nmol CYP. All results are presented as means of duplicate experiments.

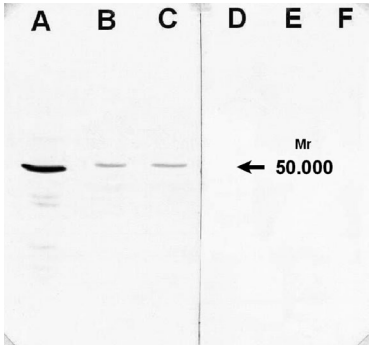

FIG. 2
Western blot of crayfish microsomal CYP. Using the affinity-purified chicken antibody against rabbit CYP2B4 (left panel), protein bands of SDS-PAGE (8.5% gel)-separated microsomal samples of phenobarbital-treated rabbit (A, D), phenobarbital-treated (B, E) and untreated (C, F) crayfish were developed. As control, a part of blot (right panel) was developed with a preimmune antibody of the same concentration (1 μg/ml)

Expression of CYP1A1 in Human Liver

There is still some conflicting evidence of the expression or inducibility of CYP1A1 protein in human liver[35–37]. Numerous studies have examined the expression of CYP1A1 in human liver. A majority of these studies reported no detectable CYP1A1 protein, as determined by immunoblotting; using conventional polyclonal mammalian antibodies or specific anti-peptide antibodies against CYP1A1 failed to detect this cytochrome P450 in human liver[35,50], and it has therefore been concluded that CYP1A1 protein is not expressed in human liver. In contrast, the results obtained with mRNA expression, protein, and activity measurements indicate that low expression levels of CYP1A1 occur in human livers[36,51,52], at less than 1% of total hepatic CYP [36,52]. Immunodetection of this CYP in human liver was clearly proved by Drahushuk et al.[52], who used a specific monoclonal antibody (mAb 1-12-3) directed against the murine fish (scap) cytochrome P450E. This antibody was shown to specifically recognize CYP1A1 in mammals[52].

Using two independent methods (immunoblotting and N-terminal sequencing), we were able to detect and quantify CYP1A1 in human hepatic microsomes. Western immunoblotting using a polyclonal antibody raised against rat recombinant CYP1A1 (highly homologous with human isoform of this enzymes, sharing 72% of sequence identity and 84% of sequence homology with orthologous human isoform) was utilized as the first method. The antibody used in the study highly cross-reacted with recombinant human CYP1A1 and only poorly with CYP1A2 (Fig. 3a, 3b). Moreover, the lack of antibody reactivity with any other human recombinant CYP expressed in Supersomes™ (CYP1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4) proved its high specificity against CYP1A (Fig. 3b). The detection limit for CYP1A1 was 0.005 pmol CYP1A1 per lane. In immunoblots (Fig. 3c), this polyclonal antibody reacted with one and/or two immunoreactive bands in most analyzed human hepatic microsomes. The high- and low-mobility bands (Fig. 3c) were assumed to be CYP1A1 and 1A2, respectively, based on the reported electrophoretic mobilities of these proteins in microsomes from human tissues[53]. To confirm that the band with lower molecular weight corresponds to human CYP1A1, N-terminal sequencing was carried out with this protein band. The bands of microsomal samples H5 and H6 were excised from the PVDF membrane and subjected to automated Edman degradation. The sequence of nine amino acids, LFPISMSAT, was identical to the residues 2–10 of the N-terminal sequence of CYP1A1. N-Terminal methionine was not found in the CYP1A1 protein band by N-terminal sequencing.

The CYP1A1 expression levels varied greatly among the different human microsomal samples (Table II), being present at <0.6% of total hepatic CYP. The range of CYP1A1 expression levels in 8 human liver samples is compa-

TABLE II
Characteristics of human hepatic microsomes and CYP1A-dependent catalytic activity

Human hepatic microsomal sample	CYP specific content[a]	CYP1A1 specific content[b]	CYP1A activity[c]
1	80	0.080	339.0
2	90	0.081	245.4
3	270	0.347	380.4
4	60	0.187	431.4
5	220	1.280	649.2
6	140	0.600	703.8
7	460	0.040	411.6
8	400	2.400	724.8

[a] The specific content was expressed as the amount of CYP (pmol, assayed as carbon monooxide adduct) per mg of total protein (BCA assay). [b] The content was expressed as the amount of CYP (pmol, determined by Western blotting) per mg of total protein (BCA assay). [c] O-Deethylation of ER in pmol/min/nmol CYP. All results are presented as means of duplicate experiments.

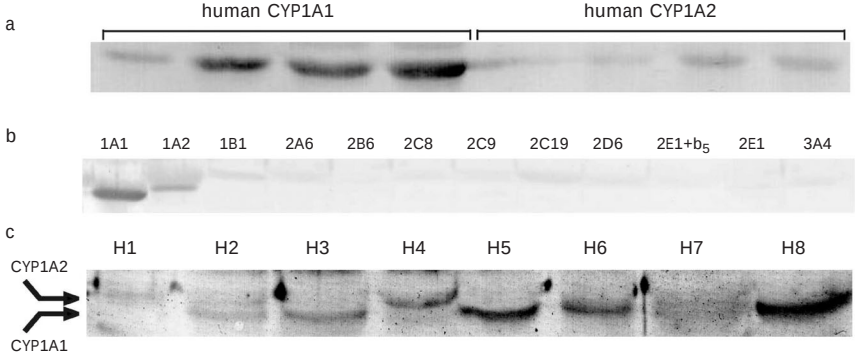

FIG. 3
Immunoblots of human recombinant CYP1A1 and 1A2 expressed in Supersomes™ (a), human recombinant CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 (b) and microsomal fraction of human liver samples H1–H8 (c). 0.1, 0.5, 1.0 and 1.5 pmol of human recombinant CYP1A1 and 1A2 (a), 1.5 pmol human recombinant CYPs (b) and 75 μg of microsomal proteins (c) were separated on SDS-PAGE (10% gel), transferred onto a PVDF membrane, and probed with the chicken anti-rat CYP1A1 affinity-purified antibody (10 μg/ml)

rable with the values recently reported[36,52]. With the same antibody, we also estimated the expression levels of CYP1A2 in all human microsomal samples. The CYP1A2 content ranged from 5 to 35 pmol per mg of microsomal protein.

CONCLUSIONS

The above examples show that chicken is able to produce antibodies with high titres against a conserved mammalian protein, cytochrome P450. Due to the larger evolutionary distance between birds and mammals, highly specific antibodies against individual CYP isoforms can be produced. Also considering the animal welfare and bio-ethics, the production of antibodies using a chicken followed by their purification from eggs is more acceptable than preparation of mammalian antisera from blood.

Thus, one can conclude that wide application of chicken antibodies in research, diagnostics and immunotherapy is a matter of time. Avian immunoglobulins will be soon accepted as a viable alternative to mammalian ones, particularly with respect to specific applications such as those discussed in this paper. Moreover, a laboratory that is ready to use non-mammalian, e.g. chicken antibodies, will be better able to adhere to stricter rules coming in the near future with regard to experimental animal handling.

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