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Monoclonal antibodies against 4-hydroxybiphenyl–UDP-glucuronosyltransferase

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Abstract—Monoclonal antibodies against purified rat liver 4-hydroxybiphenyl UDP-glucuronosyltransferase were developed using the hybridoma technology. In immunoblot analysis the antibodies specifically reacted with purified 4-hydroxybiphenyl–UDPGT but not with other purified UDPGT enzyme fractions. One single band was detected with microsomes of rat liver and small intestine but not with microsomes of kidney and lung. The reactive protein was also found in dog and human liver microsomes. It could be shown that there was no increase of immunoreactive protein after pretreatment with phenobarbital or 5,6-benzoflavone. This supports the hypothesis that more than one 4-hydroxybiphenyl–UDPGT exist in rat liver which are differently inducible.

Glucuronidation is a major pathway in phase II metabolism. Heterogeneity of UDP-glucuronosyltransferases (UDPGT*) has been shown by purification [1–4] and molecular cloning experiments [5–8].

In 1979, Bock *et al.* [9] classified several forms of UDPGT (EC 2.4.1.17) in rat liver mainly on differential induction by either 3-methylcholanthrene ("GT-1") or by phenobarbital ("GT-2"), "GT-2" catalysing the glucuronidation of morphine, 4-hydroxybiphenyl (4-HB) and chloramphenicol.

Firstly the "GT 2" could be further separated by Puig and Tephly [10] who purified an enzyme form that specifically glucuronidates morphine. In 1988, we reported on the purification of a UDPGT from 5,6-benzoflavone-pretreated rats that glucuronidated 4-HB but not morphine [11]. Styczynski *et al.* [12] purified a UDPGT conjugating 4-HB, 4-methylumbelliferone and *p*-nitrophenol. The latter two enzyme forms are probably identical.

While polyclonal antibodies are commonly used in enzyme research, monoclonal antibodies (MABs) against UDPGTs are still rare. Peters *et al.* [13] and van Es *et al.* [14] reported on the generation of MABs against human liver microsomal UDPGT. In 1988, a MAB specific for 4-nitrophenol–UDPGT could be prepared in our laboratory by immunizing mice with the purified enzyme form [15].

In the present study MABs were developed against purified 4-HB–UDPGT and characterized by immunoblot analysis.

Materials and Methods

Microsomes. Male Wistar rats, weighing 200–250 g, were pretreated with 5,6-benzoflavone (40 mg/kg i.p. for 4 days) or with phenobarbital (0.1% in drinking water for 6 days).

Microsomes from rat liver, lung, kidney and small intestine, and from liver of rabbit, dog and man were prepared as described by Falany and Tephly [1].

Enzyme purification. 4-HB–UDPGT was purified according to von Meyerinck *et al.* [2] by chromatofocusing utilizing a pH gradient from 9.2 to 5.0 and by subsequent affinity chromatography on UDP–hexanolamine-Sepharose 4B using a UDP–glucuronic acid step gradient (0.1, 0.25, 0.5 and 2.5 mmol/L). A UDPGT conjugating preferentially morphine was obtained with the same chromatofocusing system at a pH value of 7.0. 17β-Hydroxysteroid–, 3α-hydroxysteroid– and *p*-nitrophenol–UDPGT were purified according to Falany and Tephly [1].

Monoclonal antibodies. Immunization of mice and preparation of MABs were performed as described previously [15].

For immunoblot analysis (for details see Ref. 15) proteins separated on SDS gels were transferred to nitrocellulose (0.45 μm, Schleicher & Schüll) and incubated with the MABs. Immunoreactive polypeptides were detected using alkaline phosphatase-conjugated anti-mouse-immunoglobulins (Dakopatts) and nitroblue tetrazolium/5-bromo-4-chloro-indolylphosphate (NBT/BCIP) as substrate.

Results and Discussion

4-HB–UDPGT eluted from the chromatofocusing column at a pH value of 6.1. The enzyme form was further purified by affinity chromatography and could be eluted from the column with 0.5 mmol/L UDPGA. In addition to the specificity described previously [11] (no reaction with morphine and hydroxysteroids) it was found that this 4-HB–UDPGT catalyses the glucuronidation of other planar phenolic substrates like *p*-nitrophenol, 4-methylumbelliferone, and hydroxy-derivatives of phenanthrene, chrysene and picene. The activity for 1-naphthol was even higher than for 4-HB.

The enzyme form purified here is probably equal to that recently described by Styczinski *et al.* [12], although both slightly differ in their isoelectric points (6.1 vs 5.5) and apparent molecular masses (56,000 vs 52,000 kDa).

The 4-HB–UDPGT was used for immunization of female Balb/c mice and MABs were generated using the hybridoma technology. The MAB secreted by clone BI8-2 were further characterized by immunoblot analysis.

Liver microsomes from variously pretreated rats and the purified 4-HB–UDPGT were electrophoretically separated, transferred to nitrocellulose and tested for immunoreactive fractions. The results are given in Fig. 1. Independently of the antigen source, MAB BI8-2 stained only one band in the 56 kDa region. Surprisingly, an increase of immunoreactive protein after phenobarbital pretreatment could not be detected. This result was further confirmed by chromatofocusing experiments which showed that there were no differences between 4-HB–UDPGTs purified from liver microsomes of phenobarbital and of 5,6-benzoflavone pretreated rats. The effect of induction was substantiated by determination and comparison of microsomal glucuronidating activities for *p*-nitrophenol, morphine and 4-HB after 5,6-benzoflavone or phenobarbital pretreatment. From these results it can be concluded that the 4-HB–UDPGT purified here is not inducible by phenobarbital. Therefore, the well known inducibility of 4-HB glucuronidation in microsomes is due to another UDPGT isoenzyme. Styczinski *et al.* [12] claim to have purified a phenobarbital inducible 4-HB–UDPGT, but did not prove their assumption by e.g. antibody or nucleic acid analysis.

It was also tested by immunoblot analysis, whether the 56 kDa protein was present in microsomal fractions of other tissues from Wistar rats and in liver microsomes from other species. Staining of one band could be obtained with

* Abbreviations: UDPGT, UDP-glucuronosyltransferase; 4-HB, 4-hydroxybiphenyl; MAB, monoclonal antibody; BCIP, 5-bromo-4-chloro-indolylphosphate; NBT, nitrobluetetrazolium.

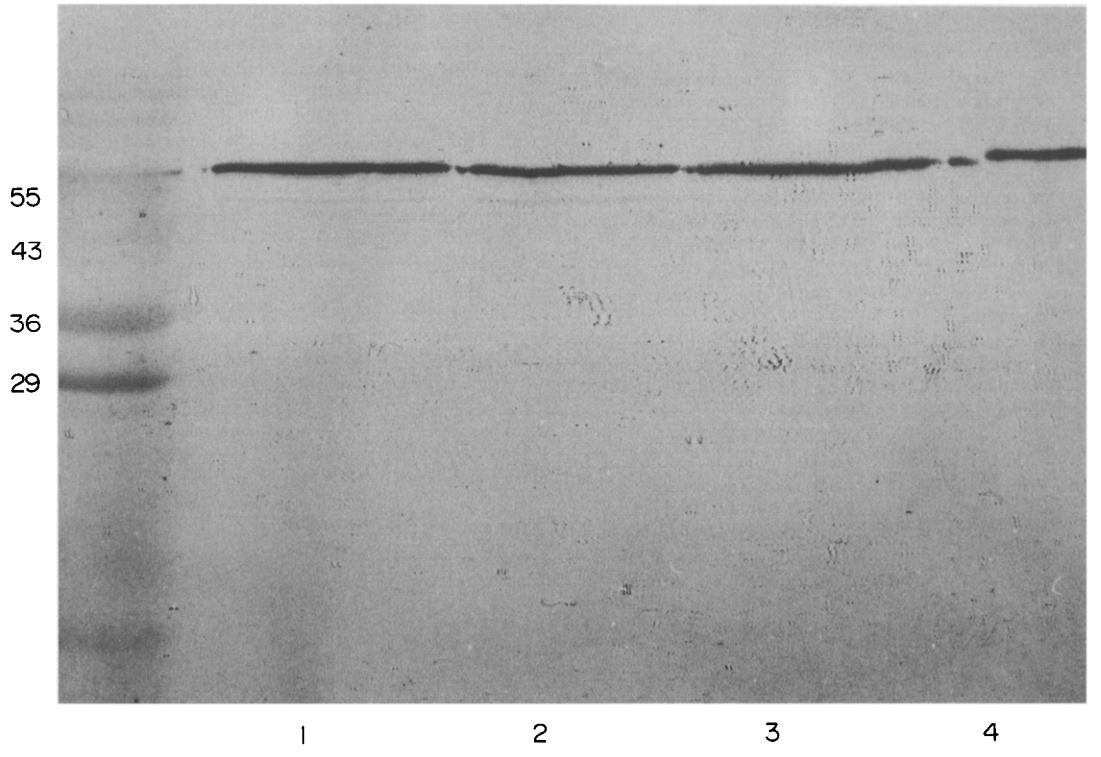

Fig. 1. Immunoblot analysis of purified 4-HB–UDPGT and of solubilized microsomes using MAB BI8-2. Following electrophoresis (12% SDS–PAGE gel) and transfer to nitrocellulose, sheets were immunostained using the 4-HB–UDPGT MAB BI8-2 and the alkaline phosphatase system (visualization was with BCIP/NBT). Lane 1 contained 5 μg of purified enzyme protein, lanes 2–4 each 20 μg of microsomal protein of differentially pretreated rats (lane 2, untreated; lane 3, phenobarbital treated; lane 4, 5,6-benzoflavone treated). Molecular mass markers ($\times 10^3$) are indicated on the left.

Table 1. Reactivity of monoclonal antibodies with different purified UDPGTs

UDPGT	Monoclonal antibody anti-4-HB-BI8-2	anti-pnpgt-67
p-Nitrophenol–UDPGT	–	+
17β-Hydroxysteroid–UDPGT	–	+
3α-Hydroxysteroid–UDPGT	–	–
Morphine–UDPGT	–	–
4-HB–UDPGT	+	–

microsomes of rat liver and intestine but not of rat lung and kidney. Whereas heavy staining could be obtained with rabbit liver microsomes, only weak reactivity was detected when dog and human liver microsomes were tested as antigen (data not shown).

MAB BI8-2 was compared with a second MAB, anti-rat-pnpgt-67, which was directed against *p*-nitrophenol–UDPGT. Both anti-bodies were tested for their reactivity with purified UDPGTs. Table 1 summarizes the results of the western blot analysis. Whereas the anti-pnpgt-67-MAB reacted with *p*-nitrophenol– and 17β-hydroxysteroid–UDPGT but not with 3α-hydroxysteroid–, morphine– and 4-HB–UDPGT, the anti-4-HB–MAB BI8-2 reacted solely with the purified 4-HB–UDPGT.

A monoclonal antibody was developed that specifically reacts with 4-HB–UDPGT but not with other UDPGT enzyme forms. It was shown that protein which was immunodetected by MAB BI8-2 is present in rat liver and small intestine but not in rat kidney and lung. It was also found in rabbit, dog and human liver. The fact that the 4-HB–UDPGT purified here, obviously is not inducible by phenobarbital raises the question, if there is another phenobarbital inducible 4-HB–UDPGT.

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