Use of monoclonal and polyclonal antibodies as structural and topographical probes for hepatic epoxide hydrolase

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Monoclonal antibodies have been prepared against rat liver epoxide hydrolase (EH), some of which gave precipitation lines on immunodiffusion against pure EH suggesting the presence of repetitive structural domains on the enzyme. Using ELISA, with polyclonal antibodies to rat and rabbit liver EH, reactivity and therefore structural similarities between EH of all species tested, including human, were observed. This was in contrast to immunodiffusion results demonstrating the limitations of the latter technique. Using monoclonal antibodies in ELISA, greatest structural similarity was between rat, mouse, and Syrian hamster EH and relatively little between rat and human. Two of the antibodies reacted with nearly all species tested and may be directed towards critical sites on the enzyme. This and most of the EH molecule would appear to be localised on the cytoplasmic surface of the endoplasmic reticulum.

Epoxide hydrolase *Monoclonal antibody* *Protein structure* *Membrane topology* *Enzyme-linked immunosorbent assay*

1. INTRODUCTION

Liver microsomal epoxide hydrolase has been investigated extensively over the past few years because of its important role in the disposition of toxic and/or carcinogenic epoxides [1]. This ubiquitous enzyme has been shown to be present in most of the organs [2] of many different animal species [3]. The enzyme has been purified in several laboratories [4–7] and some studies have demonstrated structural and catalytic similarities between the enzyme isolated from the rat and that found in human liver [7–12]. The extent of structural analogy between the rat enzyme to that present in species other than human has been limited to immunochemical cross-reactivity measured by immunodiffusion. The recent development of enzyme-linked immunosorbent assays (ELISA), together with the ability to make, site-specific, monoclonal antibodies has greatly enhanced the potential of antibodies as structural probes. Here, we have used these techniques to investigate and compare the structure and localization of epoxide hydrolase in hepatic microsomes and cytosol prepared from various animal species. Most of the polyclonal antibodies obtained to date were not inhibitory to epoxide hydrolase. The possibility that some monoclonal antibodies may be effective inhibitors was also tested.

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; IgG, immuno-γ-globulin; ABTS, 2,2-azino-bis-(3-ethyl)-benzothiozolin-6-sulphonic acid; i.p., intraperitoneal; PVC, polyvinylchloride

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2. MATERIALS AND METHODS

Hepatic microsomal and cytosolic fractions were prepared from male animals of the following species: Sprague Dawley rats (200 g), ZE1 mice (25 g), New Zealand White rabbits (2.5 kg, inbred strain), guinea pigs (300 g), Syrian hamsters (100 g) and Chinese hamsters (40 g).

In addition microsomes were prepared from monkey liver and human liver samples. Epoxide hydrolase from rat liver was purified to apparent homogeneity by the method of Bentley and Oesch [4]. Rabbit liver epoxide hydrolase was purified to apparent homogeneity by the method of Timms, Guenthner and Oesch (in preparation). Polyclonal antisera to these proteins were raised in goats [13]. Monoclonal antibodies to rat liver epoxide hydrolase were prepared from mice using standard methods summarized by Burger et al. [14]. Antibody-secreting cells were injected i.p. into BALB/c mice and the induced ascites fluid collected and stored in aliquots at −70°C. ELISA tests were carried out using conventional procedures [15]. Control samples for each determination using preimmune IgG or an unrelated monoclonal antibody were run. Epoxide hydrolase activity was measured using styrene oxide as substrate as reported in [16] but in the absence of Tween-80 as described in detail [8], one unit of activity being the amount of enzyme required to hydrate 1 nmol styrene oxide/min [4]. The inhibition of the epoxide hydrolase activity by the monoclonal or polyclonal antibodies was investigated by incubating the pure enzyme (4 units) with 25 μl of the concentrated culture medium from cells secreting antibody or with the ascites fluid diluted 1:10 (25 μl) or with polyclonal IgG (25 μl, stock 10 mg/ml) for 30 min at 4°C prior to the addition of the assay buffer and the [^{3}H]styrene oxide substrate [16]. Control incubations were run using culture medium, control ascites or control IgG.

Double immunodiffusion analysis was run by the Ouchterlony method [17]. Where microsomal samples were used, the protein was first solubilized with sodium cholate (1 mg/2 mg protein). A 5 μl solution containing 10 units of epoxide hydrolase was used per well. The IgG concentration was 10 mg/ml (5 μl). The 1% agarose gels contained 0.2% Emulgen 911. Protein was determined by the Lowry method [18].

Peroxidase-conjugated, anti-goat IgG in rabbit and peroxidase-conjugated, anti-mouse IgG in rabbit were from Dynatech (Plochingen). Freund's adjuvants were from Difco Labs (Heidelberg), ABTS was from Serva (Heidelberg) and polyethylene glycol 4000 was from Merck (Darmstadt). [^{3}H]Styrene oxide was synthesized as in [16]. All other chemicals were obtained from commercial sources.

3. RESULTS AND DISCUSSION

Monoclonal antibodies were produced against rat liver microsomal epoxide hydrolase. ELISA-positive clones (24) were selected for further investigation. The antibodies produced by all of the clones were tested by immunodiffusion against the pure enzyme and 4 of these antibodies gave precipitation lines: MZ-FC4, MZ-JC3, MZ-DG2 and MZ-HC3, 3 of which are shown in fig.1. Precipitation of a molecule by a single monoclonal antibody, in contrast to conventional antisera, requires the presence of repetitive determinants or structurally similar molecular domains on the antigen. This suggests the presence of repeating structural domains on the epoxide hydrolase molecule. Verification is needed as to whether this is so or whether it is an artifact. The fact that only 4 of the reactive antibodies gave precipitin lines, tends to

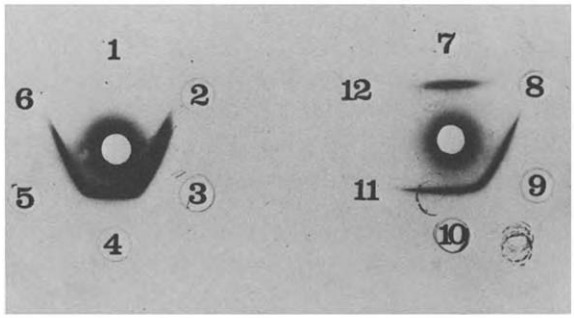

Fig.1. Ouchterlony analysis of monoclonal antibodies to rat liver epoxide hydrolase. The centre wells contained 10 μg epoxide hydrolase. The other wells contained the following ascites samples: (1) MZ-AD3 (clone B_8); (2) MZ-AD3 (clone G_8); (3) MZ-FC4 (clone B_1); (4) MZ-FC4 (clone B_8); (5) MZ-JC3 (clone E_1); (6) buffer; (7) MZ-HC3 (clone B_3); (8) MZ-HC3 (clone D_1); (9) MZ-JC3 (clone B_5); (10) MZ-JC3 (clone C_{11}); (11) buffer; (12) buffer.

rule out artifacts associated with the analysis procedure. Whether these regions are important in the catalytic activity of the enzyme is under investigation. The hepatic microsomal epoxide hydrolase activity in the various animal species used in this study is shown in fig.2. Styrene oxide was used as a representative substrate of microsomal epoxide hydrolase activity. As observed in previous studies [3], the mouse had the lowest microsomal epoxide hydrolase activity and the highest value was measured in the human sample which was 25-fold higher than in the mouse.

Various assays were used to compare the extent of structural analogy between the microsomal and cytosolic epoxide hydrolases present in different species, immunodiffusion, ELISA and monoclonal antibodies. In the first instance, the reactivity of conventional antisera prepared by immunization of goats with rat or rabbit epoxide hydrolase was tested against enzyme from various species by immunodiffusion. The antiserum to the rat enzyme showed marked reactivity and gave lines of identity with solubilized microsomes from rat, mouse and Syrian hamster. Reactivity was also observed with guinea pig microsomes, however a line of identity was not obtained. No reactivity was observed with the primate, rabbit, Chinese hamster or human microsomes. A lack of reactivity with human epoxide hydrolase has been reported by Lu and Levin et al. [19,20]. However, Guengerich et al. [10] have reported immunochemical similarities between these enzymes. Antibodies to the purified rabbit enzyme only reacted with the purified antigen and rabbit liver microsomes. On the basis of the above data, there are significant differences between the structure of various epoxide hydrolases.

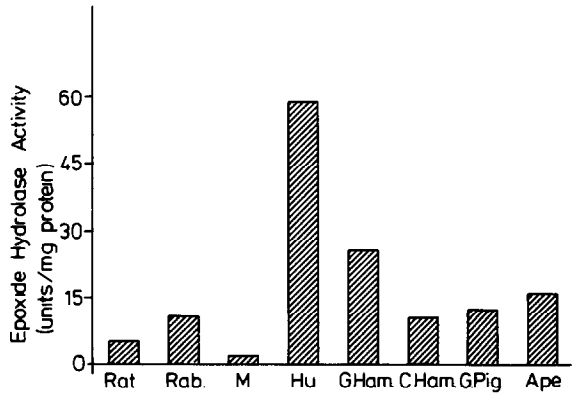

Fig.2. Epoxide hydrolase activity in liver microsomal samples prepared from various animal species using styrene oxide as substrate: Rab, rabbit; M, mouse; Hu, human; G.Ham, golden hamster; C.Ham, Chinese hamster; G.Pig, guinea pig. Experimental conditions are referred to in section 2.

The antibody to the rat enzyme also reacted with cytosolic fractions from the rat and also from the Syrian hamster and mouse (not shown). The rabbit antibody did not react with any of the cytosol fractions. Experiments are in progress to determine whether this reaction is with cytosolic epoxide hydrolases. The cytosol used did not hydrate styrene oxide at a detectable level so it is unlikely that the reactivity was due to microsomal contamination. In [21] we could not detect any reactivity of an antibody to microsomal epoxide hydrolase with the cytosolic fraction. This anomaly may be explained in that a different antibody preparation was used here. Wang et al. [22] have shown that antibodies to human liver microsomal epoxide hydrolase react with a form of the cytosolic enzyme. ELISA is an extremely sensitive assay and was used here as an alternative method for investigating structural analogy between epoxide hydrolases. A calibration curve of the extent of antibody binding against the concentration of rat epoxide hydrolase present was linear for solutions containing up to 2.5 μg epoxide hydrolase/ml (1.3 units/ml), after which concentration some deviation from linearity was observed. The detection limit was for solutions of approximately 0.1 μg/ml. Both rat and rabbit antibodies reacted in ELISA with all the species tested (table 1), indicating antigenic and therefore structural analogy between all epoxide hydrolases, and demonstrating that they may originate from a common ancestral gene. These data are not in agreement with the immunodiffusion results presented here and those of other workers. This is probably due to the higher sensitivity of ELISA compared with Ouchterlony analysis, and care should be taken when immunodiffusion assays are used as the sole evidence for lack of structural similarities. The finding that epoxide hydrolase can be precipitated by a single monoclonal antibody shows that immunodiffusion is also a poor method for demonstrating extensive structural similarities and is exemplified here by the reactivity

Table 1

Comparison of the reactivity of polyclonal antibodies to rat or rabbit epoxide hydrolase with liver microsomes from various animal species

	Rat EH (1 unit)	Rabbit EH (0.5 units)	Rat	Rabbit	Human	Ape	Guinea pig	Hamster (Syrian)	Hamster (Chinese)	Mouse
Anti-rat										
− cholate	0.32	0.08	0.29	0.20	0.15	0.18	0.12	0.24	0.27	0.26
+ cholate	0.27	0.05	0.44	0.19	0.16	0.16	0.11	0.14	0.01	0.07
Anti-rabbit										
− cholate	0.08	0.55	0.21	1.15	0.08	0.22	0.33	0.13	0.07	0.05
+ cholate	0.17	0.61	0.13	1.20	0.08	0.24	0.33	0.13	0.11	0.08

The stock solutions were: antibodies, 0.01 mg IgG/ml, microsomes containing about 2 μg epoxide hydrolase (1 unit). Solubilization was with 0.16% sodium cholate (+ cholate). Other details are given in [15]. Rat EH and rabbit EH are the purified enzymes. The experiment was repeated 3 times with similar results

of antibodies to the rat enzyme with guinea pig microsomes on Ouchterlony analysis but a low level of reactivity on ELISA. This is substantiated by the data obtained using monoclonal antibodies (see later). The extent of reactivity in the ELISA run at constant units of epoxide hydrolase varied with the species and is a measure of differences either in enzyme structure or in the accessibility of the antigen in the microsomal membrane. The latter possibility can be essentially ruled out because in the majority of cases solubilization of the microsomal membrane did not lead to a change in the extent of reaction. In some instances, for example with the rat, the interaction increased (table 1), indicating the exposure of buried antigenic sites by the solubilization procedure. In some cases, cholate caused a decrease in the peroxidase reaction; the reason for this is unclear. The extent of reaction in ELISA using the rat antibody indicated most structural similarity between the rat, mouse and Syrian hamster enzymes. By this criterion, these 3 proteins appear to be extremely similar. The largest differences from the rat were with the primate, human and guinea pig enzymes. The rabbit only reacted well with the rabbit enzyme and rabbit liver microsomes; however, it would appear to have most structural similarity with the guinea pig and least with the human enzymes. The rabbit antibody reacted poorly with the rat enzyme. However, the rat antibody reacted well with the rabbit enzyme, demonstrating similarities but possibly some significant structural differences. The low reactivity of the rabbit antibody with rat, mouse and hamster microsomes would be in agreement with the structural similarity between the latter proteins. The 13 monoclonal antibodies to the rat epoxide hydrolase with the highest titre were checked for reactivity with hepatic microsomes from various animal species (table 2). The experiment was repeated three times with the same results. A reaction was considered positive if the value obtained was 150% of a control sample run with a monoclonal antibody from another source. The number of reacting antibodies depended on the species tested. The highest number of positive reactions was with the Syrian hamster and mouse microsomal preparations, in agreement with the results using the polyclonal antibodies. That not all antibodies were positive suggests certain differences between these and the rat enzymes. Only a few of the antibodies reacted with guinea pig, ape or human microsomes. This is in contrast to various reports of extensive structural analogy of the rat with the human enzymes [7,10,11]. In [8] we demonstrated close similarities in structure-activity relationships between epoxide hydrolase in rat and man. Whether the monoclonal antibodies which react with both the rat and human enzymes are related to the catalytic properties of the enzymes remains to be investigated. The pattern of reactivity against the epoxide hydrolases of various species gives an indication of whether the anti-

Table 2

Reactivity in ELISA of monoclonal antibodies to rat liver epoxide hydrolase with microsomal samples from various species

Clone no.	Rat	Mouse	Guinea pig	Hamster (Syrian)	Hamster (Chinese)	Rabbit	Primate	Human
MZ-JB2	+	−	+	+	+	+	−	−
MZ-AD3	+	+	−	+	+	+	−	−
MZ-JC10	+	+	+	−	−	+	−	−
MZ-HC3	+	+	−	+	+	+	+	+
MZ-BG4	+	−	−	−	−	−	−	−
MZ-JC4	+	+	−	+	−	−	−	−
MZ-EF10	+	+	−	−	+	−	+	+
MZ-JB4	+	−	−	−	−	−	−	−
MZ-DD11	+	−	−	+	−	−	−	−
MZ-BD9	+	+	−	+	+	+	−	−
MZ-FC4	+	+	+	+	+	+	−	−
MZ-JC3	+	+	+	+	+	−	+	+
MZ-DG2	+	+	+	+	+	+	+	+

Liver microsomal samples from the species shown above containing 10 units of epoxide hydrolase/ml were used. Of this solution, 20 μl was taken per assay. Other details are given in section 2

bodies used are to different antigenic sites. For example, it is conceivable that clones MZ-AD3 and MZ-BD9 are the same antibody (table 2). However, by this criterion most of the antibodies used appear to be to different antigenic sites.

The data shown in table 2 were obtained with intact microsomal vesicles. On solubilization of the microsomes with sodium cholate or Emulgen 911, the reactivity of the antibodies was essentially unaltered (in some cases an inhibition of reactivity was measured) which suggests that a large proportion of the epoxide hydrolase molecule is exposed on the membrane surface. This finding is confirmed by the data in table 1.

Table 3

Effect of monoclonal antibodies to rat epoxide hydrolase on the hydration of styrene oxide

Clone no.	Epoxide hydrolase act. (nmol/min)	Control (%)	Clone no.	Epoxide hydrolase act. (nmol/min)	Control (%)
MZ-JB2	1.40	99	MZ-DD11	0.89	63
MZ-AD3	1.32	94	MZ-BD9	1.32	93
MZ-JC10	1.06	75	MZ-EB7	0.92	65
MZ-HC3	1.10	79	MZ-FC4	1.16	82
MZ-FC5	1.21	85	MZ-GG6	1.30	92
MZ-HE3	1.10	77	MZ-HC4	0.99	70
MZ-BG4	1.25	88	MZ-JC3	0.96	68
MZ-JB4	1.08	76	MZ-JB3	1.23	87
MZ-EF10	1.40	98	MZ-JB6	1.14	80
MZ-JB4	1.25	88	MZ-DG2	0.86	60
MZ-HC5	1.15	81			

Epoxide hydrolase 1.42 units was incubated for 30 min at 4°C in the presence of 20 μl concentrated IgG-containing fraction from the monoclonal cell culture medium. Total volume was 200 μl in 0.1 M Tris–HCl (pH 8.5). Following incubation, [^{3}H]styrene oxide was added and the sample incubated for 10 min at 37°C. Other conditions were as in [8]

In table 3, 21 monoclonal antibodies were tested for their ability to inhibit the hydration of styrene oxide. None of the antibodies proved to be an effective inhibitor although a few did inhibit up to 40%. It is of interest to note that of those that did inhibit, MZ-DG2 and MZ-JC3 reacted in almost all species and gave precipitin lines on Ouchterlony analysis. These antibodies may be directed at determinants located on critical sites of the enzyme molecule.

In conclusion, epoxide hydrolase may well contain repeating structural units. There are structural similarities between microsomal epoxide hydrolases of different species but also significant differences. As the substrate specificities of these enzymes are similar, the differences do not appear to be critical factors in determining activity, and the activity may be dependent on the presence of certain common peptides through all species. A large proportion of the enzyme molecule appears to be exposed on the cytosolic surface of the endoplasmic reticulum.

The application of monoclonal antibodies should prove extremely valuable in elucidating further the structural, functional and topographical aspects of this enzyme.

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