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MONOCLONAL ANTIBODIES TO RAT LIVER MICROSOMAL CYTOCHROME b_5

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Abstract—Hybridomas obtained by the fusion of spleen cells from rat cytochrome b_5-immunized mice with mouse mycloma cells produced five groups of monoclonal antibodies (MAbs) with three mouse immunoglobulin subtypes: IgG1, IgG2b and IgM. All of the MAbs bound strongly to rat cytochrome b_5 as measured by radioimmunoassay (RIA). Four clones of MAbs were also strongly immunoreactive with cytochrome b_5 when tested by Western blotting, but only one of the MAbs (1-39-2) weakly immunoprecipitated cytochrome b_5 in an Ouchterlony double-immunodiffusion test. Two of the MAbs partially inhibited cytochrome b_5-mediated NADH cytochrome *c* reduction catalyzed by liver microsomes (24–36%). Expression of immunodetectable cytochrome b_5 was highest in the liver, next highest in the kidney, and quite low in the other tissues examined with MAb 1-17-1 by Western blotting. This MAb recognized homologous cytochrome b_5 of human liver microsomes and in homogenates of TK$^-$ cells infected with recombinant vaccinia virus encoding human cytochrome b_5. These MAbs to cytochrome b_5 will be useful for the identification, quantification, and purification of cytochrome b_5 from animal and human tissues, and for understanding its role in cytochrome P450 catalyzed drug metabolism and carcinogen activation with respect to tissue, organ and individual differences.

Cytochromes P450 are key components of the mixed-function oxidases which metabolize numerous xenobiotics such as drugs, chemical carcinogens, and endobiotics, for example fatty acids, steroids and prostaglandins [1, 2]. The basic mixed-function oxidase system requires the interaction of cytochrome P450 with NADPH–cytochrome P450 reductase in a lipid environment [3]. With several cytochromes P450 [4], including the forms designated PB-1 (2C6) [5], 2c/RLM5 (2C11) [6] and P450$_{ac/et}$ (2E1) [7] for rats and LM4 (2B4) for rabbits, cytochrome b_5 may participate in the monooxygenase pathway by serving in electron transfer from NADPH–cytochrome P450 reductase to cytochrome P450 [4]. To study the functional role of each cytochrome P450, we are preparing a library of monoclonal antibodies (MAbs‡) to the individual cytochromes P450. Heretofore we have reported on the preparations and properties of MAbs to eight different forms of cytochrome P450 [8–16]. In this study, we have successfully prepared MAbs to a purified rat liver microsomal cytochrome b_5. These MAbs recognize the cytochrome b_5 of rat, rabbit and human liver, and the cytochrome b_5 expressed by vaccinia viruses containing human cytochrome b_5 cDNA.

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‡ Abbreviations: CFS$^-$, serum free culture fluid concentrate; HAT, hypoxanthine, aminopterin and thymidine; HT, hypoxanthine and thymidine; MAbs, monoclonal antibodies; PBS, phosphate-buffered saline; and RIA, radioimmunoassay.

MATERIALS AND METHODS

Preparation of microsomes, purified cytochrome P450 and recombinant human cDNA cytochrome b_5 *expressed by vaccinia viruses in cell homogenates.* Microsomes were prepared from the livers of neonatal (3–4 days), young (100–125 g) and adult (230–260 g) male and female Sprague–Dawley rats as described previously [17]. The human liver microsomes were a gift from Dr. F. P. Guengerich [18]. Recombinant vaccinia viruses containing human cytochrome b_5 cDNA, which code human cytochrome b_5 [19], were constructed by inserting the cDNA into a vaccinia virus cDNA expression vector by a method described previously [20, 21]. Infection of human TK$^-$ 143 cells with this recombinant virus in culture results in the production of 0.3 nmol cytochrome b_5/mg cell lysate proteins [21]. Rat cytochrome b_5 [22] which was used as an immunogen and rabbit cytochrome b_5 were purified as described previously [23]. Rat cytochromes P450 were obtained as follows: 1A1 (P450c/BNF-B), 1A2 (P450d/ISF-G), 2B1 (P450b/PB-B/PB-4), 2D1 (P450 UTH), and 3A (P450 PCN-E) from Dr. F. P. Guengerich [24]; 2C11 (P450h/2c/RLM5 from Dr. J. B. Schenkman [25]; and 2E1 (P450j/P450ac/et) from Dr. C. S. Yang [7]. Fish cytochrome P450E, 1A1 family was obtained from Dr. J. J. Stegeman [26].

Immunization of mice and production of hybridomas. Female Balb/c mice were immunized with rat cytochrome b_5 and the primed spleen cells from five mice were used for hybridization with myeloma cells, SP 2/0. Hybridomas were selected in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum, 10% horse

serum, 50 μg/mL gentamicin, 100 μm hypoxanthine (H), 0.4 μM aminopterin (A), 100 μM thymidine (T) (HAT medium). The hybridomas were screened by radioimmunoassay (RIA) and cloned three times to obtain independent hybrid clones and ensure monoclonality. The final clones were grown in hypoxanthine and thymidine (HT) medium in which only aminopterin was depleted from the above HAT medium, and transferred to serum free culture medium to prepare serum free culture concentrates (CFS^-). The hybridomas were also grown in the HT medium, harvested, resuspended in phosphate-buffered saline (PBS; GIBCO) and inoculated into mice for ascites preparations (5,000,000 cells/mice). The CFS^- and ascites served as the source of the MAbs. Their preparation was described in detail in a previous report on MAb to cytochrome P450 preparation [11].

Immunobiochemical methods. RIA, subtyping of mouse immunoglobulins, Ochterlony double-immunodiffusion and Western blotting which was followed by a 10% polyacrylamide gel electrophoresis were carried out as described in the previous reports on MAbs to cytochromes P450 [11–13]. Protein concentrations in CFS^-, ascites and microsomes were measured by the method of Lowry *et al.* [27].

Assays for the effect of MAbs on cytochrome b_5 *activity.* Liver microsomes (5–10 μg) isolated from Sprague–Dawley adult male and female rats were preincubated for 20–30 min at 20–22° with CFS^- (100 μg) as a source of MAbs or purified MAbs (0–4 μg protein) in 0.5 mL of 0.3 M KP_i buffer (pH 7.7) with 0.5 mg/mL cytochrome *c*. MAbs were purified from ascites through a Rec-Protein G column (Zymed) according to the manufacturer's protocol. Cytochrome *c* reduction was measured spectrophotometrically (ΔA_{550nm}) at 33° after initiating the reaction by the addition of NADH (final concentration = 0.1 mg/mL) [28].

RESULTS

Identification and classification of MAbs. Table 1 shows the general characteristics of the MAbs to cytochrome b_5 characterized in this study. The binding data of serum antibodies from b_5 immunized rats to cytochrome b_5 were the mean values measured by the RIA when the sera were diluted 100 times in PBS. The binding of serum antibodies to cytochrome b_5 was 10 times greater than that of the unimmunized mice sera.

The spleen cells of the immunized mice were hybridized with the myeloma cells SP 2/0. The five independent hybridomas formed produced MAbs to cytochrome b_5. One produced IgG2b, two produced IgG1, and two produced the IgM subtype of mouse immunoglobulins. The MAbs produced by the hybridomas bound to cytochrome b_5 4–30 times more than the binding of the nonspecific MAb, NBS 1-48-5. Four of the MAbs also showed strong immunoreactivity with cytochrome b_5 by Western blotting analyses. Only one of the MAbs, 1-39-2, yielded a weak immunoprecipitin reaction measured by Ouchterlony double-immunodiffusion analyses. We also observed a weak immunoprecipitin reaction with serum polyclonal antibodies as shown in Table 1 and Fig. 1.

Effects of MAbs on cytochrome b_5 *activity.* To observe the effects of MAbs to cytochrome b_5 on cytochrome b_5 activity, the influence after preincubation of the MAbs on rat liver microsomal cytochrome b_5-dependent NADH cytochrome *c*

Table 1. General characteristics of MAbs to rat liver microsomal cytochrome b_5*

Antibody	Mouse Ig subtype	RIA Binding to b_5 (cpm)	Immunoreaction to b_5	
			Western blot	Precipit. (Ochterlony)
Polyclonal antibodies				
Normal serum		390	−	−
Mouse serum Ab/b_5		4300	+	+
MAbs				
NBS 1-48-5 (control)	IgG2a(k)	370	−	−
B_5 1-38-1	IgG2b(k)	2210	+	−
B_5 1-39-2	IgG1(k)	6710	+	+
B_5 1-82-1	IgM(k)	1180	+	−
B_5 1-84-2	IgM(k)	4870	−	−
B_5 1-17-1	IgG1(k)	9020	+	−

*Ninety-six well microtiter plates were precoated with purified cytochrome b_5 (1 μg in 100 μL PBS), and antibody binding was determined by [^{35}S]methionine incorporated antimouse IgG(k) binding (cpm). Serum antibodies were diluted 100 times and used in the RIA and without dilution in Ouchterlony double-immunodiffusion. Serum free culture fluid concentrates (25×) were used as the source of MAbs for the studies on mouse immunoglobulin subtyping, Western blotting and immunoprecipitation. Culture fluids were used for the binding of MAbs to cytochrome b_5.

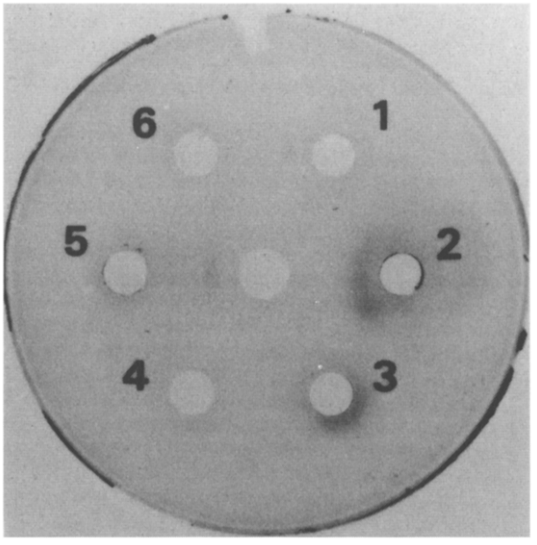

Fig. 1. Double-immunodiffusion analysis of Ig secretion and MAbs to cytochrome b_5. The center well contained 15 μL of purified cytochrome b_5 (0.3 mg/mL) and the outer wells 1, 2, 3, 4, 5, and 6 contained 15 μL of serum free culture concentrates, NBS 1-48-45 (7.5 mg/mL), mouse polyclonal serum antibody to cytochrome b_5 (about 60 mg/mL), MAb 1-17-1 (3.9 mg/mL), MAb 1-38-1 (12.1 mg/mL), MAb 1-39-2 (11.4 mg/mL) and MAb 1-84-2 (3.6 mg/mL).

Table 2. Effects of MAbs to rat liver microsomal cytochrome b_5 on NADH-dependent cytochrome *c* reductase activity

Reagent	Inhibitory effect (%)
Antimycin A (200 μM)	77
MAbs	
NBS 1-48-5 (control)	7
HyHel-9	4
B_5 1-38-1	15
B_5 1-39-2	0
B_5 1-82-1	0
B_5 1-84-2	24
B_5 1-17-1	26

The effects of MAbs on cytochrome b_5 activity were carried out by monitoring cytochrome *c* reduction spectrophotometrically at 550 nm, initiating the reaction by the addition of NADH. Microsomes, cytochrome *c* and serum free culture concentrates as the source of MAbs were preincubated at room temperature for 20 min as described in Materials and Methods.

reductase was monitored after preincubation of two sources of MAbs. When 10 μg of liver microsomes was preincubated with 100 μg of CFS⁻, 0–26% inhibition of the cytochrome *c* reduction was observed, as shown in Table 2. Addition of antimycin A inhibited 77% of the reaction, assuring that the

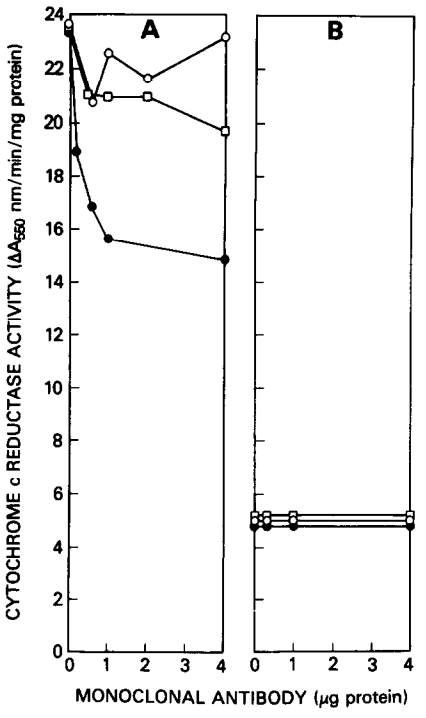

Fig. 2. Effect of MAbs to rat liver microsomal cytochrome b_5 on cytochrome *c* reductase activity. Adult male rat liver microsomes (5 μg) were incubated with MAbs for 30 min at room temperature, and the reductions of cytochrome *c* were measured spectrophotometrically at 550 nm in the presence of NADH (A) or NADPH (B) as described in Materials and Methods. The rates of cytochrome *c* reduction (ΔA_{550nm}/min/mg) were plotted against the concentrations of MAbs (1–4 μg protein). MAbs [HyHel-9 (□); 1-82-1 (○); 1-17-1 (●)] in ascites were purified through a Rec-Protein G column (Zymed) and used in this experiment.

reaction was cytochrome b_5-dependent NADH reduction system. When the experiment was repeated with the highest inhibitory MAb, 1-17-1, after purification, as shown in Fig. 2A, the rate of cytochrome *c* reduction catalyzed by liver microsomes from adult male rats was decreased by increasing the levels of purified MAb 1-17-1. This reduction reached 36% with 4 μg of the purified MAbs. In contrast, the effect of MAb 1-82-1 was variable and essentially not significant. Non-specific MAb HyHel-9 showed 16% inhibition. Similar results were observed with adult female rat liver microsomes (data not shown). As a control for the potential non-specific effect of MAb 1-17-1 on microsomal cytochrome *c* reduction, we assessed the inhibitory effect of this MAb on microsomal NADPH–cytochrome *c* reductase activity, a cytochrome b_5-independent reaction that is catalyzed by NADPH–cytochrome P450 reductase. No inhibition was observed (Fig. 2B).

Western blot analysis of cytochrome b_5. To study the b_5 species-specificity of the inhibitory MAb 1-17-1, microsomal proteins from rat and rabbit liver and cell homogenates prepared from uninfected

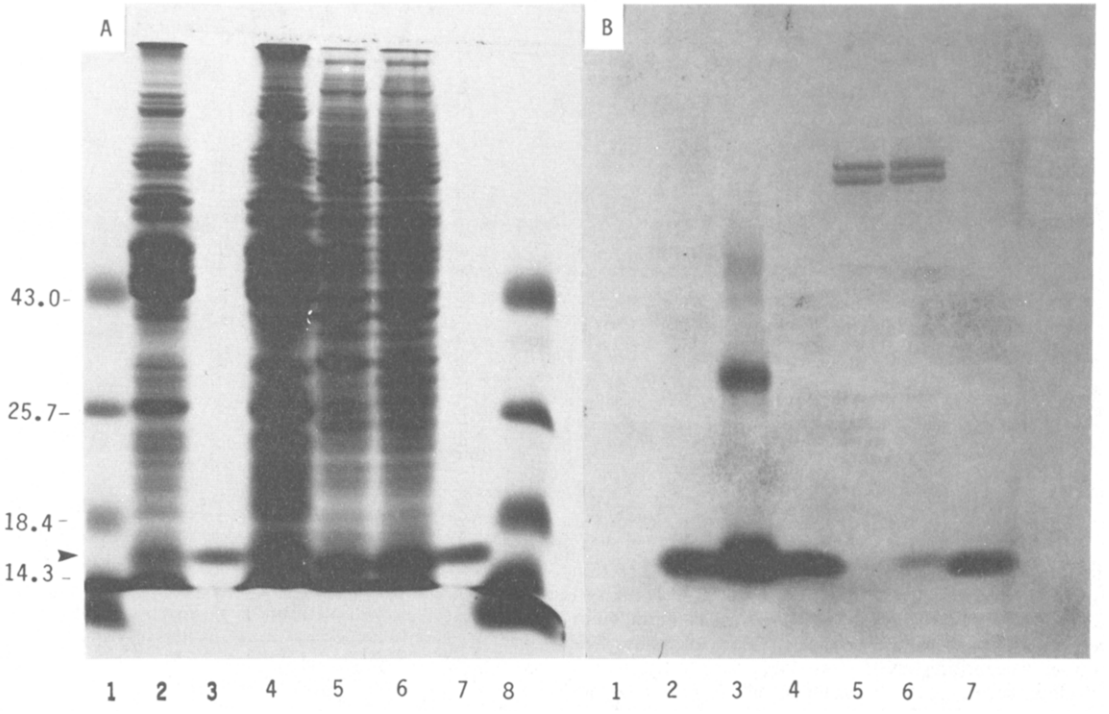

Fig. 3. Analysis of microsomal and TK⁻ cell homogenate proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by Coomassie Blue staining (A) and Western immunoblotting with MAb 1-17-1 (B). Lanes 1–8 contained molecular weight standard proteins (5 μg), adult male rat liver microsomes (50 μg), purified rat liver cytochrome b_5 (2.5 μg), adult female rat liver microsomes (50 μg), TK⁻ cell homogenates (200 μg), homogenates of TK⁻ cells that were infected with vaccinia viruses carrying human cytochrome b_5 cDNA, purified rabbit cytochrome b_5 (2.5 μg), and molecular weight standard proteins (5 μg).

TK⁻ cells or cells infected with vaccinia viruses containing human cytochrome b_5 cDNA were analyzed by Western blotting (Fig. 3). These analyses indicated a high degree of specificity of MAb 1-17-1 for b_5 from rat (lanes 2 and 4), human (lanes 5 and 6) and rabbit (lane 7) cytochrome b_5.

In the case of the cell homogenates, a pair of unidentified higher molecular weight proteins that were present both in the control TK⁻ cells and the b_5-expressing cells were also observed (lanes 5 and 6). The specificity of MAb 1-17-1 was further examined by Western blotting of liver microsomes from neonatal, young and adult male and female rats. As shown in Fig. 4, cytochrome b_5 was detected in liver microsomes from all the rats with an increased amount detected in the older rats. Cytochrome b_5 was also detected in microsomes from five human individual liver samples (lanes 7–11) as well as in the homogenates of TK⁻ cells infected with vaccinia containing cDNA from human cytochrome b_5 (lane 13).

Although unlikely, we checked possible cross-reactivity of MAb 1-71-1 with a number of rat cytochromes P450 and with rat and rabbit NADPH–cytochrome P450 reductases by Western blotting. Eight forms of cytochromes P450 (1A1, 1A2, 2B1, 2C11, 2D1, 2E1, 3A and fish 1A1) and two different forms of NADPH–cytochrome P450 reductase were compared to the immunoreactivity of rat and rabbit cytochrome b_5. As expected, MAb 1-17-1 showed immunorecognition of rat and rabbit cytochrome b_5 but no cross-reactivity of the cytochromes P450 or the NADPH-cytochrome P450 reductase examined (data not shown).

MAb 1-17-1 was also useful for the testing or organ-specific expression of b_5. As shown in Fig. 5, the expression of b_5 was the highest in liver, and the degree of expression in decreasing order was liver, kidney, lung, testis, male spleen, female spleen, brain, heart, and esophagus. We also observed unidentified highly cross-reactive proteins of ~25,000 daltons in brain and ~43,000 in heart. The nature of the proteins was unknown. Similar results were observed with CFS⁻ (Fig. 5A) and ascites fluids (Fig. 5B), demonstrating that MAbs obtained in CFS⁻ and ascites are equally effective in the immunological reactions. Since the degree of cytochrome b_5 expression varied in different organs as determined by Western blotting, densitometric analysis was made for the quantitative assessment taking the blot density with liver as 100%. As shown in Table 3, the densitometric analysis revealed that the levels of cytochrome b_5 expressed in the extrahepatic tissues examined were highest in kidney (19–30% of liver levels) and were low, but detectable in lung, spleen, testis and brain (1–4% of liver levels). Cytochrome b_5 levels were too low to quantitate in heart.

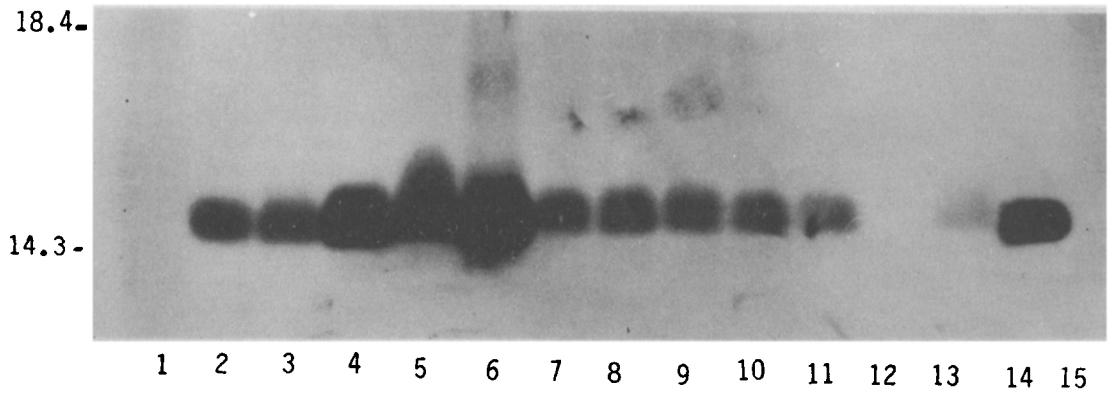

Fig. 4. Western blot analysis of cytochrome b_5 with MAb 1-17-1. Sample proteins were resolved by SDS-polyacrylamide gel electrophoresis and incubated with MAb 1-17-1 for immunoblotting as described in Materials and Methods. Lanes 1 and 15 contained molecular weight standard proteins (5 μg); lanes 2–5, neonatal, male young, male adult, and female adult liver microsomes (50 μg); lane 6, rat liver microsomal cytochrome b_5 (2.5 μg); lanes 7–11, human liver microsomes 15B, 18G, 21S, 22A, and 23A (50 μg); lanes 12 and 13, TK^- cell homogenates are homogenates of TK^- cells infected with vaccinia viruses carrying human cytochrome b_5 cDNA; and lane 14, purified rabbit cytochrome b_5.

DISCUSSION

The participation of cytochrome b_5 in the monooxygenase pathway serving in electron transfer from NADPH–cytochrome P450 reductase to cytochrome P450 [4–7] and the significant role it plays in physiological functions [29] have been reported. The cytochrome b_5-mediated enhancement of mixed-function monooxygenation was also clearly demonstrated utilizing recombinant vaccinia viruses carrying cytochrome b_5 [21]. Since cytochrome P450 plays a key role in the metabolism of xenobiotics and some endobiotics, MAbs to cytochrome P450 are useful for identifying and determining cytochrome P450 without the necessity of purification of cytochrome P450 by conventional biochemical procedures. MAbs have proven useful for the identification and analysis of cytochromes P450 from various mammalian sources [17, 30–32]. The role of cytochrome b_5 in the cytochrome P450-mediated metabolism of xenobiotics could be an additional factor for individual differences in the metabolism of xenobiotics. Inhibitory MAbs to cytochrome b_5 would be useful for identifying precisely which P450 requires the participation of cytochrome b_5 for maximum activity. The MAbs to cytochrome b_5 described in this study are specific and should prove useful for the identification and quantification of cytochrome b_5 in animal and human tissues as well as for its immunopurification.

It appears that the two interactions between MAbs and cytochrome P450 and the MAbs described here and cytochrome b_5 are different. The molecular weight of cytochrome b_5 is about 16,800 daltons and that of P450 is about 50,000 daltons. We frequently observed immunoprecipitin reaction of MAbs with cytochrome P450 but the immunoprecipitin reaction with cytochrome b_5 was very weak, as shown in Fig.

Table 3. Quantitation of rat cytochrome b_5 by MAb 1-17-1 in different organs by densitometric tracing of Western blots

Sex	Organ	Blot density (peak area, mm^2 at A_{600nm}/ 100 μg microsomal protein)	Relative b_5 level (%)
Male	Liver	120,000	100
	Lung	1,100	1
	Heart	0	0
	Kidney	23,000	19
	Spleen	5,000	4
	Testis	3,400	3
	Brain	3,700	3
Female	Liver	94,000	100
	Lung	3,200	3
	Heart	0	0
	Kidney	28,000	30
	Spleen	3,100	3
	Brain	2,200	2

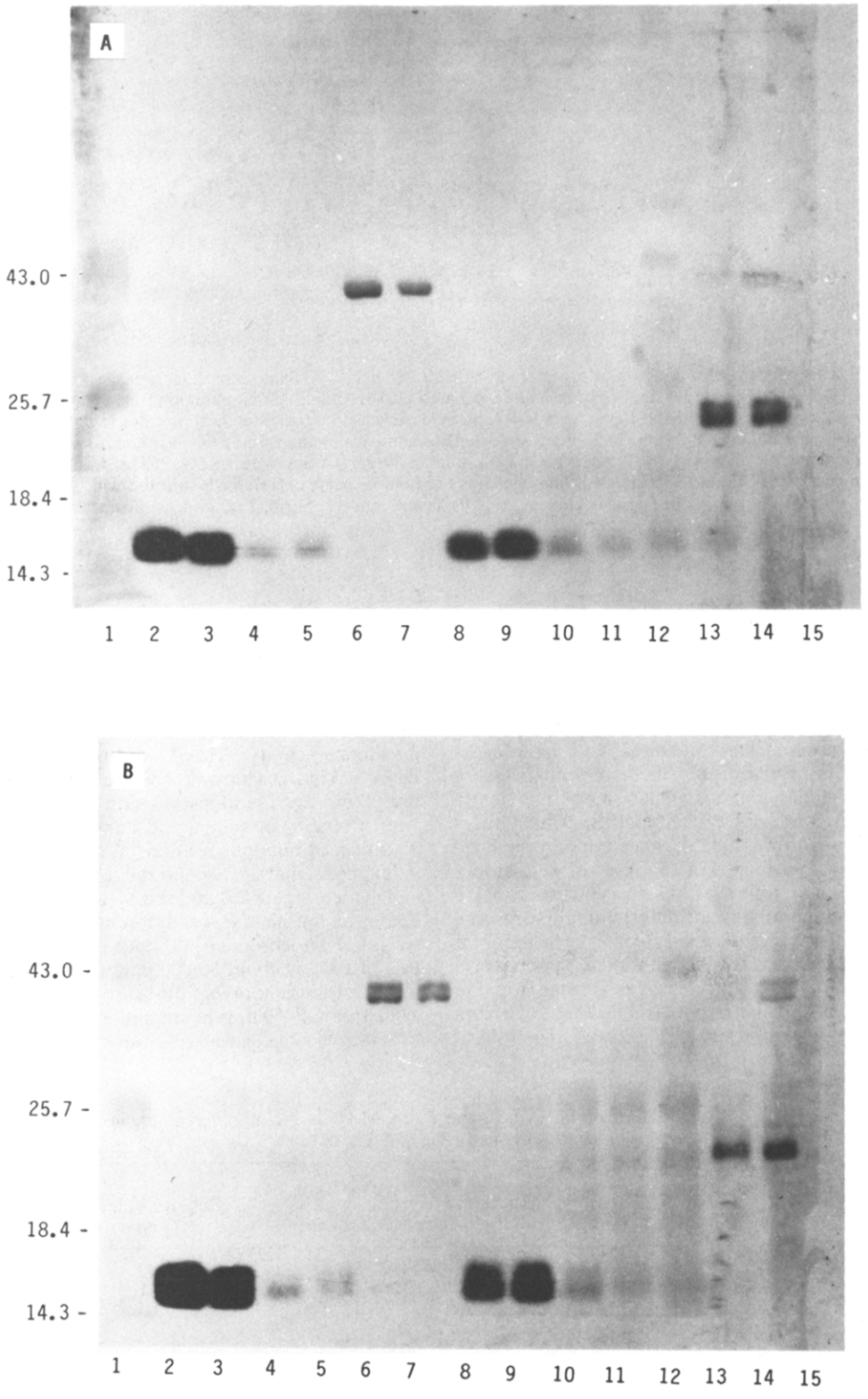

Fig. 5. Western blot analysis of organ-specific expression of cytochrome b_5 in rats. Western blotting was with MAb 1-17-1 in serum free culture fluid concentrates (50 μg/mL in PBS, Fig. 5A) or ascites (500 μg/mL, Fig. 5B) as described under Materials and Methods. Lane 1, molecular weight standards; lanes 2–11, microsomal proteins of adult male and female rat liver (50 μg), male and female lung (100 μg), male and female heart (100 μg), male and female kidney (100 μg) and male and female spleen (100 μg); lane 12, testis (100 μg); lanes 13 and 14, adult male and female brain (100 μg); and lane 15, adult male esophagus (100 μg).

1. This was true even with polyclonal anti-b_5 antibodies. The MAb 1-71-1 recognized rabbit and human cytochrome b_5 as well as rat cytochrome b_5, indicating that these contained common epitopes. The specificity would be useful for identification, immunopurification and quantification of cytochrome b_5 from different tissues and organs, if proper standards could be provided. Even though the MAbs do not have strong inhibitory activity, it certainly indicates that cytochrome b_5 is involved in the cytochrome *c* reduction, and MAb 1-17-1, which gives 36% inhibition, may be useful in identifying cytochromes P450 that require cytochrome b_5 for maximum activity. It would also be of interest to know the properties of the unknown cross-reacting proteins of high molecular weights in the microsomal preparations of heart and brain. MAb 1-17-1 cross-reacted with the proteins of molecular weight ~43,000 daltons and brain proteins of molecular weight ~25,000 daltons when male and female rat heart and brain microsomes were tested. The cross-reactivity of the proteins with MAb 1-17-1 should be clarified for its utility for the immunohistochemistry in brain and heart. Increased amounts of cytochrome b_5 were detected in the young and adult rat livers as compared to neonatal rat liver with the suggestion that the expression of cytochrome b_5 is developmentally regulated. A further interesting observation is the high content of cytochrome b_5 in kidney as previously described [29]. The detection with a MAb to rat cytochrome b_5 of a protein expressed by human cytochrome b_5 cDNA not only confirms that the protein was epitopically identical to the rat and rabbit b_5 but also further indicates that the vaccinia cDNA expression system produces proteins with characteristics similar to those of the animal cytochrome b_5.

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