J. steroid Biochem. Vol. 23, No. 6A, pp. 1013–1021, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

MONOCLONAL ANTIBODIES AGAINST BOVINE ADRENAL CYTOCHROME P-450$_{11\beta}$ AND CYTOCHROME P-450$_{SCC}$. THEIR ISOLATION, CHARACTERIZATION AND APPLICATION TO IMMUNOHISTOCHEMICAL ANALYSIS OF ADRENAL CORTEX

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(*Received* 25 *March* 1985)

Summary—Seven monoclonal antibodies directed against bovine adrenocortical cytochrome P-450$_{11\beta}$ were isolated. They were found to be immunoglobulins each having distinct affinity to the antigen. Five clones had IgG1 heavy chains, whereas the other two had IgG2b heavy chains. All the clones had κ light chains. All the monoclonal antibodies recognized a protein among mitochondrial proteins of bovine adrenal cortex, whose mol. wt, 50,000, was the same as that of cytochrome P-450$_{11\beta}$. Among the monoclonal antibodies isolated, monoclonal antibody 258 recognized a protein of Mr 50,000 in rat adrenal mitochondria, a protein of Mr 47,000 in pig adrenocortical mitochondria, a protein of Mr 50,000 in guinea pig adrenal mitochondria, a protein of Mr 55,000 in rabbit adrenal mitochondria and a protein of Mr 50,000 in human adrenocortical mitochondria. These results suggest that each of these proteins, having a mol. wt around 50,000, recognized by monoclonal antibody 258 is very likely to be the cytochrome P-450$_{11\beta}$ of adrenocortical mitochondria of each animal species. Monoclonal antibody 42718 inhibited by 50% the steroid hydroxylation activity of cytochrome P-450$_{11\beta}$. In addition to the monoclonal antibodies against cytochrome P-450$_{11\beta}$, three monoclonal antibodies directed against bovine adrenocortical cytochrome P-450$_{SCC}$ were also isolated and characterized. Immunohistochemical staining of bovine adrenal cortex with the use of these two kinds of monoclonal antibodies revealed that the contents of both cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{SCC}$ were greater in the zona fasciculata and zona reticularis than in the zona glomerulosa. Electron microscopical observation of immunoperoxidase-stained preparations confirmed the presence of these cytochromes in the mitochondria.

INTRODUCTION

Cytochrome P-450$_{11\beta}$ of adrenocortical mitochondria is known to catalyze both 11β- and 18-hydroxylations of 11-deoxycorticosterone [1–3], either 11β- or 19-hydroxylation of 18-hydroxy-11-deoxycorticosterone (18(OH)DOC) [4–6], and 18-hydroxylation of corticosterone [3]. Recently we have reported that this cytochrome can also catalyze aldosterone biosynthesis from corticosterone or 18-hydroxycorticosterone, and the biosynthetic rate is finely modulated by the presence of phospholipids [7, 8]. These versatile catalytic properties of cytochrome P-450$_{11\beta}$ raise an intriguing question as to the structure of the active center of this enzyme. How can the active center accommodate a variety of steroids as substrates, and what functional residue in the active center determines the point of hydroxylation on the steroid skeleton? Are all these functions controlled by environmental factors such as membrane phospholipids surrounding the cytochrome? In order to answer these interesting questions, an attempt was made to raise and isolate monoclonal antibodies (MAbs) directed against cytochrome P-450$_{11\beta}$. Unlike polyclonal antibodies, a MAb is a chemically defined homogeneous reagent which has a discrete binding affinity for a single epitope. Use of a MAb for immunopurification would enable us to purify only an antigen having the single epitope. Moreover, if we could isolate MAbs showing inhibitory activity toward the enzyme action, kinetical analysis of the enzyme action in the presence of these MAbs would reveal a mechanism regulating the multi-site specificity of the enzyme.

In this study, seven MAbs directed against cytochrome P-450$_{11\beta}$ were isolated and characterized as to their specificity to the antigen. One MAb recognized an epitope shared by the cytochromes P-450$_{11\beta}$ from various animal sources. Another MAb showed inhibitory activity toward 11β- and 18-hydroxylations

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of 11-deoxycorticosterone catalyzed by the cytochrome.

Also reported in this paper are the isolation and characterization of three MAbs directed against cytochrome P-450$_{SCC}$ of bovine adrenocortical mitochondria. Cytochrome P-450$_{SCC}$ catalyzes a side-chain cleavage reaction of cholesterol, which is believed to be a rate-determining step in steroidogenesis [9–11]. Application of these two kinds of MAbs directed against cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{SCC}$ for immunohistochemical characterization of bovine adrenal cortex is also described.

EXPERIMENTAL

Chemicals

Polyethylene glycol 4000 was purchased from Nakarai Chemicals, Japan. Pristane (2,6,10,14-tetramethylpentadecane) was a product of Aldrich. Horseradish peroxidase–conjugated F(ab')$_2$ fragment of sheep anti-mouse Ig was obtained from Amersham. Rabbit anti-mouse Ig antibodies specific to immunoglobulin classes and subclasses and horseradish peroxidase–conjugated goat anti-rabbit IgG (heavy and light chain specific) were purchased from Zymed Laboratories, Inc., U.S.A. Steroids and bovine serum albumin (BSA) were products of Sigma. NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase (yeast) were purchased from Oriental Yeast Co., Japan. All other chemicals of the highest purity available were obtained from commercial sources.

Enzymes

Cytochrome P-450$_{11\beta}$ was purified to homogeneity from bovine adrenocortical mitochondria according to the method described by Momoi *et al.*[5]. The specific content of cytochrome P-450 of the purified material was 10.8 nmol P-450/mg protein. Steroid hydroxylation activities of the purified cytochrome P-450$_{11\beta}$ were determined as described previously [3–6]. The cytochrome catalyzed both 11β- and 18-hydroxylations of 11-deoxycorticosterone at the rates of 110 nmol corticosterone formed/min/nmol P-450 and 7.6 nmol 18(OH)DOC formed/min/nmol P-450, respectively. Cytochrome P-450$_{SCC}$ was purified to homogeneity from bovine adrenocortical mitochondria as described previously [12]. Purification of adrenodoxin and NADPH-adrenodoxin reductase was performed as reported [13, 14].

Preparation of adrenal mitochondria

Preparation of adrenal mitochondria from various animal species was performed as described previously [15].

Production and purification of monoclonal antibodies

The method of production of MAbs was essentially the same as those described by Goding[16] and Okuno *et al.*[17] with some modification. Inbred Balb/c mice were immunized by intraperitoneal injection with 100 μg of purified cytochrome P-450$_{11\beta}$, or cytochrome P-450$_{SCC}$, in Freund's complete adjuvant (0.5 ml). One month later a booster injection was given intraperitoneally, or intreavenously via the tail vein, with 10–50 μg of the antigen dissolved in phosphate-buffered saline (PBS, 10 mM sodium phosphate-150 mM NaCl, pH 7.4, unless otherwise specified) without the adjuvant. The spleens of the mice were removed aseptically on the third day after the booster injection. The spleen cells were mixed with 8-azaguanine resistant mouse myeloma cells SP2/0-Ag14 (SP2) at a cell number ratio of between 1:1 and 10:1 in the presence of 50% polyethylene glycol 4000 dissolved in Dulbecco's modified Eagle's medium (DMEM). Fused cells suspended in DMEM supplemented with 10% fetal calf serum (FCS) were plated onto 96-well microtest plates so that each well contained 10^5 myeloma cells in 100 μl. The cells were maintained at 37°C in an atmosphere of 98% air and 2% CO_2. On the next day, selective medium containing DMEM, 10% FCS, 0.1 mM hypoxanthine, 0.4 μM aminopterine and 16 μM thymidine was added to the wells, and the culture was continued for a further 10 days. Then the selective medium was changed to that containing DMEM, 10% FCS, 0.1 mM hypoxanthine and 16 μM thymidine. After 1 week, the medium was again changed to DMEM containing 10% FCS. The cells were periodically inspected by microscopy, and when visible colonies appeared in the wells, the culture fluids from viable hybridomas were screened for the presence of antibodies against cytochrome P-450$_{11\beta}$, or cytochrome P-450$_{SCC}$, by means of an enzyme-linked immunosorbent assay (ELISA) as described below. The hybridomas producing antibodies were cloned twice by a limiting-dilution method on a feeder layer of spleen cells from normal mice. Those clones which continued to secrete the antibodies were expanded to large-scale cultures. Balb/c mice, which had been primed by intraperitoneal injection of 0.5 ml of pristane 10 days before, were injected with 10^6 cells of the cloned hybridomas. Approximately 3–5 ml of ascites fluid was obtained from each mouse after 10–14 days. Immunoglobulin was purified from the fluid by ammonium sulfate fractionation followed by exclusion chromatography on a column of Sephacryl S-300 in a buffer of 0.1 M sodium bicarbonate–0.5 M NaCl (pH 8.6).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted according to the method described by Schenk and Leffert[18] with some modification. One hundred μl of PBS, pH 8.0, containing an antigen, cytochrome P-450$_{11\beta}$ (30 ng) or cytochrome P-450$_{SCC}$ (30 ng), was placed in each well of a polystyrene microtiter well (Flow Laboratories, Inc.). The microtiter plate was incubated overnight at 4°C or for 3 h at room temperature, during which time the antigen was adsorbed to the plate. After the

incubation, the PBS was removed and the plate was washed twice with PBS containing 0.05% Tween 20. In order to block residual binding sites on the plate, 200 μl of PBS containing 1% BSA was added to each well and the plate was incubated for 1 h at room temperature. The plate was washed as described above, and then 50 μl of hybridoma culture fluid or purified antibodies appropriately diluted with PBS containing 0.1% BSA was added to each well. Incubation of the plate was carried out for 1 h at 37°C. The plate was washed 3 times as described above and then 50 μl of horseradish peroxidase–conjugated F(ab')$_2$ fragment of sheep anti-mouse Ig (diluted 1000 times in PBS) was added to each well. Incubation was performed for 2 h at 37°C or overnight at 4°C. The plate was washed as described above and then 100 μl of substrate solution containing 1 mM 2,2-azino-di-(3-ethylbenzthiazoline sulfonate) [ABTS] and 0.003% H_2O_2 was added to each well. After an appropriate incubation time (usually 10–30 min) at 37°C, the peroxidase reaction was terminated by addition of 50 μl of 0.1 mM citric acid containing 0.01% (w/v) sodium azide per well. Absorbance of the reaction mixture was recorded at 405 nm with a Corona microplate photometer (MTP-22).

Determination of mouse immunoglobulin classes and subclasses was also performed by an ELISA. Hybridoma culture fluids containing MAbs were placed in the antigen-coated microtiter wells. The plate was incubated for 1 h at room temperature, followed by 3 washes with PBS containing 0.05% Tween 20. Fifty μl of a solution containing class- and subclass-specific rabbit anti-mouse Ig was added to each well and the plate was incubated for 1 h at room temperature. After washing of the plate, 50 μl of horseradish peroxidase–conjugated goat anti-rabbit IgG (heavy and light chain specific) was added to the well. After further incubation for 1 h at room temperature followed by washing of the plate, the substrate solution containing 1 mM ABTS and 0.003% H_2O_2 in 100 μl was added to the wells and the plate was kept at room temperature until the contents of positive wells became dark.

Immunoblot analysis (Western blotting)

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of cytochrome P-450$_{11\beta}$, cytochrome P-450$_{SCC}$ and adrenal mitochondria from various animal species was carried out according to the method described by Laemmli[19]. The proteins on a slab gel were electrophoretically transferred to a nitrocellulose membrane. The electrotransfer was performed at 30 V for 3 h in a buffer containing 25 mM Tris–HCl, 192 mM glycine, 20% methanol and 0.05% SDS, pH 8.3 [20]. Specific antigenic proteins on the nitrocellulose membrane were detected by an enzyme-immunostaining method using anti-cytochrome P-450 MAbs with horseradish peroxidase–conjugated second antibody as described by Domin *et al.*[21] with some modification. Nonspecific binding sites on the nitrocellulose membrane were blocked with 3% BSA. The membrane was incubated with 0.1 mg/ml of each MAb for 15 min at 25°C and then incubated with horseradish peroxidase–labeled anti-mouse Ig (F(ab')$_2$ fragment) for 15 min at 25°C. For detection of peroxidase activity, the membrane was stained with 4-chloro-1-naphthol and H_2O_2. The apparent relative molecular weight (Mr) of the immunochemically stained protein was estimated by comparing its mobility with those of standard proteins which were electrophoresed on the same gel and stained with Coomassie brilliant blue R.

Other analytical methods

Protein concentration was determined by the method of Lowry *et al.*[22] using crystalline BSA as the standard. The concentration of cytochrome P-450 was estimated as described by Sato *et al.*[2], using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the absorbance difference at 450 nm of the CO-binding reduced enzyme. Concentrations of adrenodoxin and NADPH-adrenodoxin reductase were determined using $A_{414} = 10\ mM^{-1}\,cm^{-1}$ and $A_{450} = 11\ mM^{-1}\,cm^{-1}$, respectively [14, 23].

Light and electron microscopic immunohistochemistry

Fresh bovine adrenal glands obtained from a slaughterhouse immediately after the death of the animal were cut into small pieces and fixed in 4% paraformaldehyde buffered at pH 7.4 with Milloning's phosphate for 12 h at 4°C.

For light microscopic immunohistochemical analysis, fixed tissues were washed in PBS containing 10–20% sucrose for 8 h. Frozen sections mounted on glass slides were treated with the MAbs (diluted at 1:100–1000) for 36–72 h at 4°C, then rinsed in PBS and finally incubated with horseradish peroxidase conjugated anti-mouse IgG for 12 h at 4°C. After rinsing in PBS, the sections were treated with a medium containing 0.005% 3,3'-diaminobenzidine (DAB) and 0.003% H_2O_2 for 5–10 min at room temperature. For electron microscopic immunohistochemical analysis, fixed tissue samples were embedded in 10% gelatin dissolved in PBS and frozen sections were made. The sections were rinsed and dipped in a solution of antibodies as described above. After incubation with DAB solution, sections were fixed in 1% OsO_4 solution, dehydrated with a graded series of ethanol concentrations and embedded in Epon epoxy resin. Ultrathin sections were cut and examined under a Hitachi H-500 type electron microscope without staining. Control sections were treated with preimmune mouse serum instead of the MAbs.

RESULTS

Characterization of MAbs directed against cytochrome P-450$_{11\beta}$

Seven hybridoma cells lines, designated as 231, 210,

Table 1. Characterization of MAbs directed against cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{SCC}$

MAb		Immunoglobulin: Heavy chain	Immunoglobulin: Light chain	Amount of MAb for 50% saturation, ng/well
Anti-P-450$_{11\beta}$	231	IgG2b	κ	12.5
	210	IgG1	κ	3.5
	42718	IgG1	κ	45.0
	218	IgG1	κ	4.3
	21	IgG2b	κ	2.5
	214	IgG1	κ	8.0
	258	IgG1	κ	9.3
Anti-P-450$_{SCC}$	65-9	IgG2a	κ	2.4
	E128	IgG2b	κ	2.3
	5D$_2$	IgG1	κ	10.6

Immunoglobulin classes and subclasses were determined using antibodies specific to mouse IgA, IgM, IgG1, IgG2a, IgG2b, IgG3, κ light chain and λ light chain as described in Experimental. Affinity of the MAbs to the antigen was determined by means of an ELISA using various amounts of purified MAbs in microtiter wells, the surface of which had been previously coated with 30 ng/well of cytochrome P-450$_{11\beta}$ or cytochrome P-450$_{SCC}$.

42718, 218, 21, 214 and 258, producing antibodies directed against bovine adrenocortical cytochrome P-450$_{11\beta}$ were selected. Antibody-containing ascites fluid was obtained by injection of pristane-treated mice with the hybridoma cells. Immunoglobulin was prepared from the ascites fluid by ammonium sulfate fractionation followed by gel filtration chromatography. Characterization of the immunoglobulin was performed by an ELISA specific for mouse immunoglobulin classes and subclasses. As shown in Table 1, 5 clones, 210, 42718, 218, 214 and 258, had IgG1 heavy chains, whereas the other two clones, 231 and 21, had IgG2b heavy chains. All the clones had κ light chains.

The affinity of each Mab to the antigen was determined by an ELISA technique. Various amounts of each MAb were placed in microtiter wells, the surface of which had been previously coated with a certain amount of cytochrome P-450$_{11\beta}$. After the incubation, the wells were washed, and sheep anti-mouse immunoglobulin antibody coupled with horseradish peroxidase was added to the wells. The extent of MAb binding to the antigen could be determined by means of the peroxidase reaction. Typical results are presented in Fig. 1, where the absorbance at 405 nm is plotted against the amount of the MAb added. Binding of the MAbs to the antigen on the plate showed a saturation curve. MAb 21 seemed to bind to the antigen more avidly than MAb 231. By analysis of these saturation curves, the amounts of these MAbs for 50% saturation were determined to be 2.5 ng/well for MAb 21 and 12.5 ng/well for MAb 231, respectively. Similar analysis gave the results shown in the third column of Table 1, which served as rough estimates of the relative affinities of the MAbs to cytochrome P-450$_{11\beta}$.

Immunoblot analysis of the specificity of MAbs directed against cytochrome P-450$_{11\beta}$

To examine the specificity of each MAb to cytochrome P-450$_{11\beta}$, immunoblot analysis was performed. Cytochrome P-450$_{11\beta}$ as well as other proteins were electrophoresed on a SDS-polyacrylamide slab gel. After the electrophoresis, the proteins on the polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane (Western blotting). The membrane was then incubated with each MAb, so that the MAb could bind to the antigen on the membrane. After washing of the membrane, the bound MAb was detected with horseradish peroxidase–conjugated F(ab')$_2$ fragment of sheep IgG specific to mouse immunoglobulin. Staining of the membrane by the peroxidase reaction revealed a band corresponding to the antigen recognized by the MAb. As shown in Fig. 2, all the MAbs recognized the purified bovine adrenocortical cytochrome P-450$_{11\beta}$ and a protein in bovine adrenocortical mitochondria which had the same molecular weight as cytochrome P-450$_{11\beta}$ (Mr 50,000). None of these MAbs recognized the purified cytochrome P-450$_{SCC}$. None of the MAbs except MAb 258 recognized any protein in rat

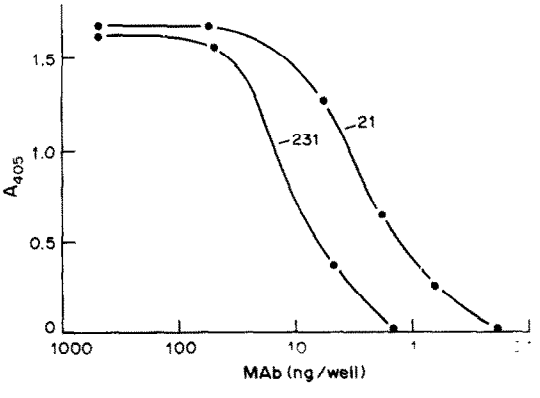

Fig. 1. Binding of MAbs 231 and 21 to cytochrome P-450$_{11\beta}$ determined by an ELISA. Serially diluted MAbs 231 and 21 were incubated in microtiter wells precoated with 30 ng of purified cytochrome P-450$_{11\beta}$. MAb bound to the antigen was determined as described in Experimental.

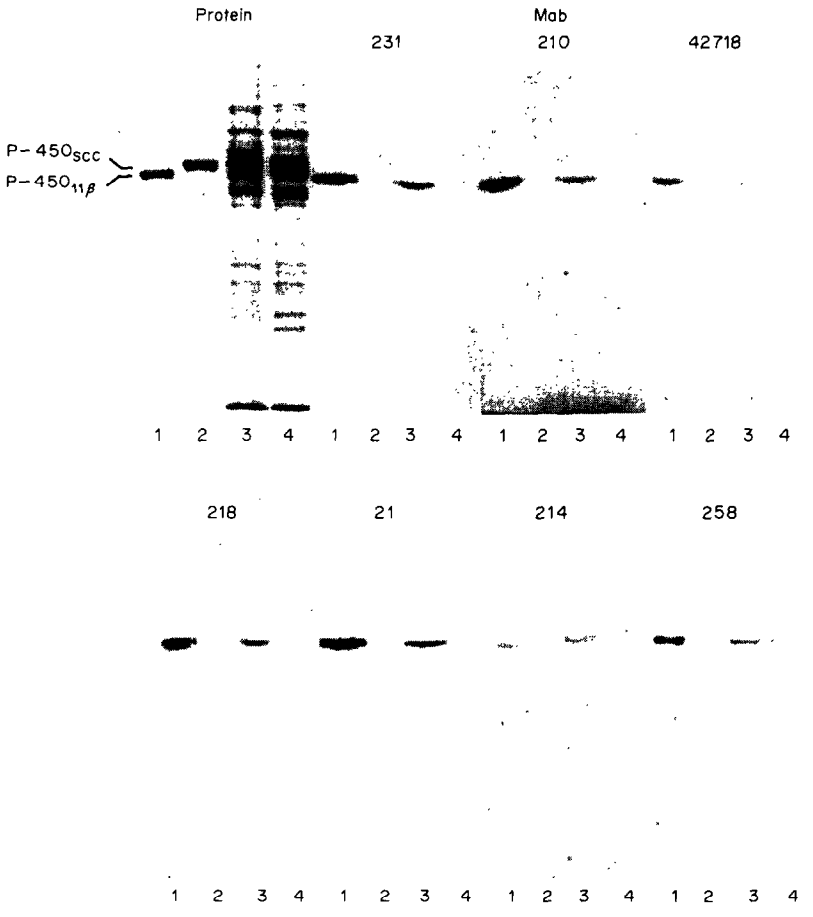

Fig. 2. Immunoblotting of SDS-polyacrylamide gel electrophoretograms using anti P-450$_{11\beta}$ MAbs. Cytochrome P-450$_{11\beta}$ (lane 1), cytochrome P-450$_{SCC}$ (lane 2), bovine adrenocortical mitochondria (lane 3) and rat adrenal mitochondria (lane 4) were electrophoresed on a SDS-polyacrylamide gel. After the electrophoresis, the proteins on the gel were electrophoretically transferred to a nitrocellulose membrane, and stained with MAbs as probes. Immunostaining was performed as described in Experimental using each MAb mentioned at the top of each set of four tracks. The four tracks at top-left show protein-staining of the slab gel with Coomassie brilliant blue R. Lanes 1 and 2 contained 1.5 μg of protein per track and lanes 3 and 4 17μg of protein per track.

adrenal mitochondria. It is interesting that MAb 258 recognized a protein having a mol. wt of 50,000 among mitochondrial proteins of rat adrenal glands (Lane 4 of the MAb 258-stained gel). It is highly likely that this protein of Mr 50,000 is the cytochrome P-450$_{11\beta}$ of rat adrenal cortex. Therefore MAb 258 seems to be an antibody directed against an epitope of cytochrome P-450$_{11\beta}$ which is present in both the rat enzyme and the bovine enzyme.

To further examine whether MAb 258 recognizes an epitope common to cytochromes P-450$_{11\beta}$ from the other animal sources, adrenal mitochondria of various animal species were examined by the immunoblotting technique using MAb 258 as a probe. As shown in Fig. 3, MAb 258 recognized, in addition to the purified bovine cytochrome P-450$_{11\beta}$, the protein of Mr 50,000 in bovine adrenocortical mitochondria and the protein of Mr 50,000 in rat adrenal mitochondria, a protein of Mr 47,000 in pig adrenal mitochondria, a protein of Mr 50,000 in guinea pig adrenal mitochondria, a protein of Mr 50,000 in human adrenocortical mitochondria, and a protein of Mr 55,000 in rabbit adrenal mitochondria. Because only one band appeared in each lane, it is conceivable that each of these proteins, having molecular weights around 50,000, recognized by MAb 258 is the cytochrome P-450$_{11\beta}$ of the adrenocortical mitochondria of the respective animal species.

Effect of MAbs on cytochrome P-450$_{11\beta}$-catalyzed reactions

Cytochrome P-450$_{11\beta}$ was incubated with each MAb in the presence of adrenodoxin for 16 h on ice. After the incubation, cytochrome P-450$_{11\beta}$ was assayed as to 11β- and 18-hydroxylation activities with 11-deoxycorticosterone as a substrate. As shown in Table 2, MAb 42718 clearly inhibited the steroid hydroxylation activities by about 50% under these

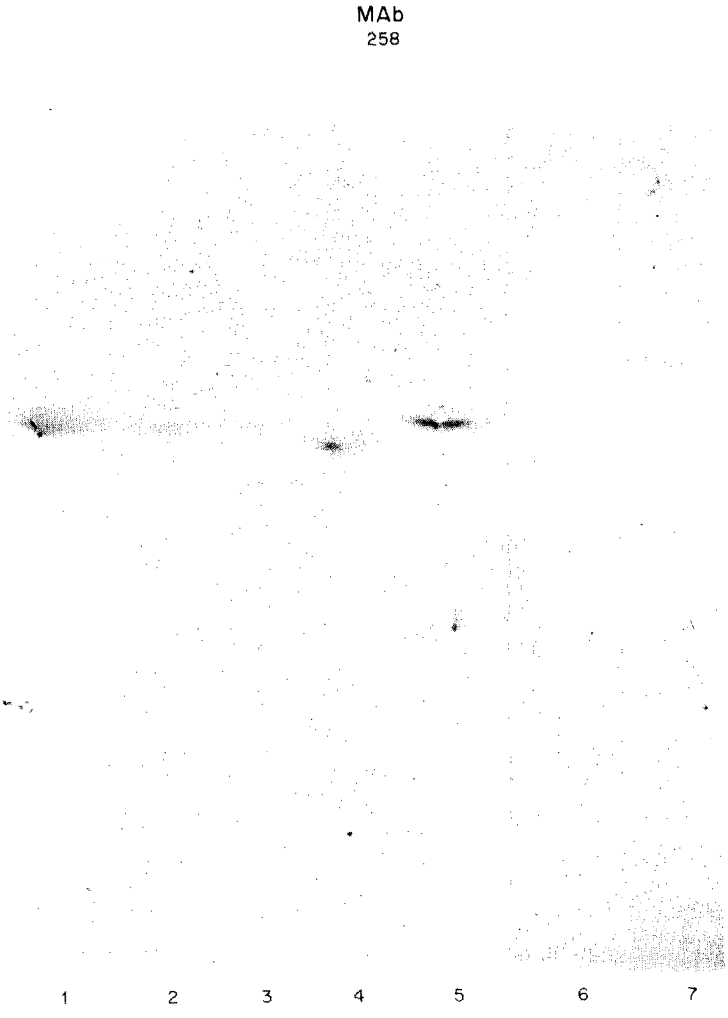

Fig. 3. Immunoblot analysis of the specificity of MAb 258 to a protein of adrenal mitochondria from various animal species. Lanes 1–7 show immunochemical staining of a slab gel of purified cytochrome P-$450_{11\beta}$ (lane 1), bovine adrenocortical mitochondria (lane 2), rat adrenal mitochondria (lane 3), pig adrenocortical mitochondria (lane 4), guinea pig adrenal mitochondria (lane 5), human adrenocortical mitochondria (lane 6) and rabbit adrenal mitochondria (lane 7). Lane 1 contained 1.5 μg of protein per track and lanes 2–7, 17 μg of protein per track.

conditions. MAb 231 had no effect on the enzyme activities. MAbs other than MAbs 42718 and 231 seemed to only slightly inhibit the steroid hydroxylation activities. Figure 4 illustrates the dose-dependency of the effect of MAb 42718 on the 11β- and 18-hydroxylation activities of cytochrome P-$450_{11\beta}$. This MAb seemed to reduce both the activities by as much as 50%, but no further inhibition was observed. When a sonicated bovine adrenocortical mitochondrial fraction preincubated with MAb 42718 under similar conditions was assayed as to 11β- and 18-hydroxylation activities, a similar extent of inhibition by MAb 42718 was observed (data not shown).

Characterization of MAbs directed against cytochrome P-450_{SCC}

Three hybridoma cell lines, designated as 65-9, E128 and $5D_2$, producing antibodies directed against cytochrome P-450_{SCC} were selected, and the MAbs were prepared from the ascites fluids of these hybridomas. Characterization of immunoglobulin subclasses of these MAbs as well as determination of their affinity to cytochrome P-450_{SCC} were performed as described for those experiments conducted on the MAbs directed against cytochrome P-$450_{11\beta}$. The results are presented in Table 1. MAb 65-9 had an IgG2a heavy chain, MAb E128 an IgG2b heavy chain, and MAb $5D_2$ an IgG1 heavy chain. All the three MAbs had κ light chains.

Immunoblot analysis of the specificity of MAbs directed against cytochrome P-450_{SCC}

Immunoblotting was performed using each anti-P-450_{SCC} MAb as a probe. As shown in Fig. 5, all the MAbs recognized the purified bovine adrenocortical

Table 2. The effect of MAbs on 11β- and 18-hydroxylase activities of cytochrome P-450$_{11\beta}$ in a reconstituted system

Addition of MAb		Hydroxylase activity nmol/min/nmol P-450$_{11\beta}$ Corticosterone formed	18(OH) DOC formed
None		100 (100)	6.6 (100)
MAb	231	101 (101)	6.9 (105)
	210	63 (63)	4.3 (65)
	42718	53 (53)	3.7 (56)
	218	72 (72)	4.7 (71)
	21	67 (67)	4.6 (70)
	214	86 (86)	5.6 (85)
	258	88 (88)	5.8 (88)

Purified cytochrome P-450$_{11\beta}$ (0.04 nmol) was incubated in 23 μl of 30 mM Tris–HCl buffer with each MAb (1.25 nmol) in the presence of 2 nmol adrenodoxin overnight on ice. After the incubation, the mixture was added to the reaction mixture containing, in a final vol of 1 ml, 30 mM Tris–HCl buffer (pH 7.4), 200 nmol 11-deoxycorticosterone, 6 nmol adrenodoxin, 1 nmol NADPH-adrenodoxin reductase, 5 μmol glucose-6-phosphate, 0.5 unit glucose-6-phosphate dehydrogenase, 4 μmol $MgCl_2$ and 100 nmol NADPH. The reaction was carried out at 37°C for 4 min. The steroids were extracted with dichloromethane and analyzed by high performance liquid chromatography as described in Experimental. "None" denotes an assay conducted in the presence of 187.5 μg BSA instead of MAb. The numbers in parentheses are the percentages of the activity in the absence of MAb.

cytochrome P-450$_{SCC}$ and a protein among mitochondrial proteins of bovine adrenal cortex whose mol. wt, 55,000, was the same as that of cytochrome P-450$_{SCC}$. None of the MAbs recognized the purified cytochrome P-450$_{11\beta}$ or any protein in rat adrenal mitochondria.

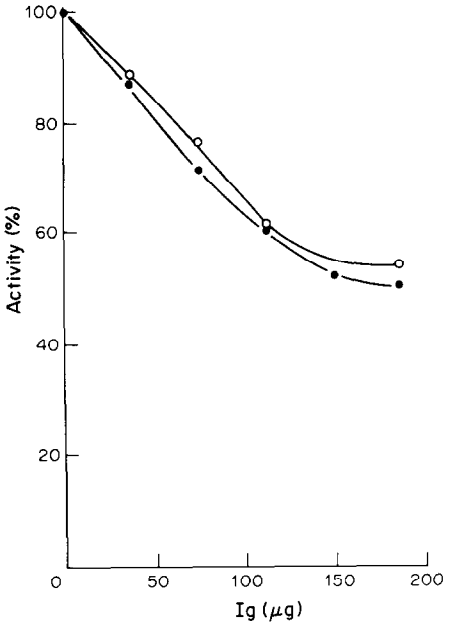

Fig. 4. Inhibition of cytochrome P-450$_{11\beta}$-catalyzed 11β- and 18-hydroxylase activites by MAb 42718. Purified cytochrome P-450$_{11\beta}$ (0.04 nmol) was incubated with increasing amounts of MAb 42718 in the presence of 2 nmol adrenodoxin for 16 h on ice. Cytochrome P-450$_{11\beta}$-catalyzed 11β- and 18-hydroxylations of 11-deoxycorticosterone were assayed in a reconstituted system as described in Experimental and in the legend to Table 2. The activities were expressed as percentages of the control (110 nmol corticosterone formed/min/nmol P-450$_{11\beta}$ and 7.6 nmol 18(OH)DOC formed/min/nmol P-450$_{11\beta}$). Closed circles, activity of 11β-hydroxylation; open circles, activity of 18-hydroxylation.

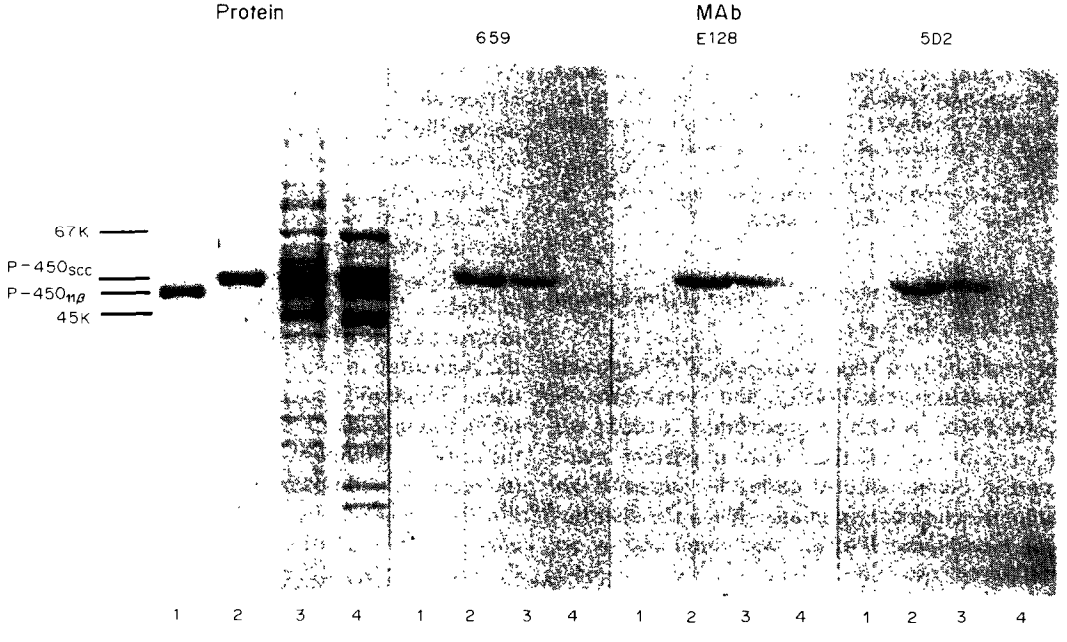

Fig. 5. Immunoblotting of SDS-polyacrylamide gel electrophoretograms with anti P-450$_{SCC}$ MAbs. Cytochrome P-450$_{11\beta}$ (lane 1), cytochrome P-450$_{SCC}$ (lane 2), bovine adrenocortical mitochondria (lane 3) and rat adrenal mitochondria (lane 4) were electrophoresed, and immunoblotting was conducted as described in Experimental and in the legend to Fig. 2. The four tracks at the left show protein-staining of the slab gel with Coomassie brilliant blue R. Lanes 1 and 2 contained 1.5 μg of protein per track and lanes 3 and 4 17 μg of protein per track.

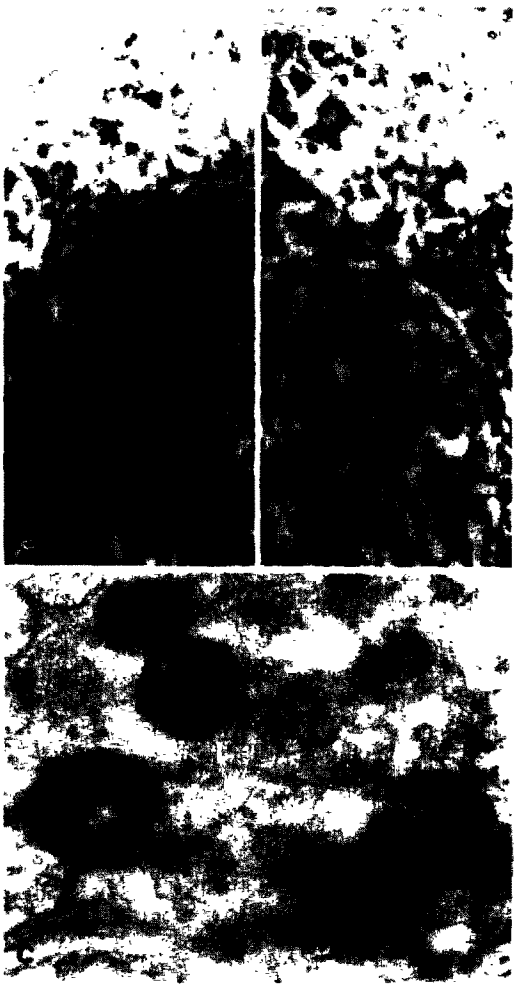

Fig. 6. Light (a,b) and electron (c) microscopic immunohistochemistry. a, c: Immunostaining with MAb 65-9. b: Immunostaining with MAb 210. a, b: Cells of the zona glomerulosa were faintly positive for both cytochrome P-450_{scc} and cytochrome P-$450_{11\beta}$, while cells of the zona fasciculata were strongly positive for both enzymes. c: Reaction products for cytochrome P-450_{scc} were localized on the inner mitochondrial membrane and matrix. a, b × 120, c × 23,000.

Application of the MAbs to immunohistochemical analysis of adrenal cortex

The availability of the two kinds of MAbs each specific to cytochrome P-$450_{11\beta}$ and cytochrome P-450_{scc} provided us with an opportunity to study the zonal distribution of these two important cytochromes in bovine adrenal cortex by means of an immunohistochemical technique. When the distribution of cytochrome P-450_{scc} was examined by an indirect immunostaining technique using MAb 65-9 and the horseradish peroxidase-labeled second antibody, intense staining was observed in parenchymal cells of zonae fasciculata and reticularis (Fig. 6a). The staining of the parenchymal cells of the zona glomerulosa was faint. No staining was observed in the capsule or medulla of the adrenal gland.

Electron microscopical observation revealed intracellular localization of cytochrome P-450_{scc} (Fig. 6c). The inner mitochondrial membrane of the parenchymal cells was intensely stained by MAb 65-9, suggesting that cytochrome P-450_{scc} was exclusively localized in the mitochondria. No other cellular membranes, such as the plasma membrane, endoplasmic reticulum or nuclear membrane, were stained. It should be noted that the extent of staining of the mitochondria in a single cell differed markedly from mitochondria to mitochondria. This phenomenon was observed by Mitani *et al.*[24] using a polyclonal antibody raised against this cytochrome.

Immunohistochemical analysis employing MAb 210 gave essentially the same results as to the distribution (Fig. 6b) and the intracellular localization of cytochrome P-$450_{11\beta}$ as those for cytochrome P-450_{scc}.

DISCUSSION

In this study, seven MAbs specific to bovine adrenocortical cytochrome P-$450_{11\beta}$ were isolated and characterized. Among these MAbs, MAb 258 was found to be an antibody able to recognize an epitope common to cytochromes P-$450_{11\beta}$ of various animal species. Application of this MAb to immunoaffinity purification of a protein of adrenal mitochondria would enable us to very easily purify cytochrome P-$450_{11\beta}$ of various animal species.

MAb 42718 was shown to inhibit cytochrome P-$450_{11\beta}$-catalyzed 11β- and 18-hydroxylations of 11-deoxycorticosterone. The extent of inhibition was similar for both reactions. These results seem to confirm the observation that a single protein catalyzes the two hydroxylations [25]. Cytochrome P-$450_{11\beta}$ has been reported to catalyze 19-hydroxylation of steroids [2, 5] and aldosterone synthesis [7] in addition to the 11β- and 18-hydroxylations. Use of MAb 42718 for kinetical analysis of these reactions is under investigation in our laboratory.

To get more insight into the mode of inhibition of steroid hydroxylations by this MAb, we tested various incubation conditions other than those described in Results. We altered the time of the incubation, the temperature of the incubation and the composition of the incubation mixture. An incubation time of longer than 12 h seemed to be required to obtain the inhibition, suggesting a possibility that the inhibition of the enzyme activity by MAb 42718 might be due to the secondary conformational change of cytochrome P-$450_{11\beta}$ triggered by binding of the MAb to the enzyme. An incubation temperature below 4°C and the presence of adrenodoxin in the incubation mixture seemed to be required because of the unstable nature of the purified cytochrome P-$450_{11\beta}$.

The attempt to raise a MAb which completely inhibits the steroid hydroxylase activity was unsuccessful. A possible reason for the failure may be that the active site of cytochrome P-$450_{11\beta}$ is buried deep in the enzyme molecule and, therefore, is not

very immunogenic. However, it is still possible that the MAb having a strong inhibitory activity might be found if the culture mediums of more hybridomas were examined.

Three MAbs specific to bovine adrenocortical cytochrome P-450$_{SCC}$ were also isolated and characterized in the present study. Using the two kinds of MAbs each specific to cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{SCC}$, we conducted immunohistochemical characterization of the bovine adrenal cortex. The results clearly indicated the presence of these 2 cytochromes in 3 zones of the adrenal cortex. However, the contents of the cytochromes seemed to differ among the 3 zones. The zonae fasciculata and reticularis were rich in the 2 cytochromes, whereas the zona glomerulosa was poor in them. Intracellular localization of these two cytochromes was also determined by an immunoelectron–microscopical study. The two cytochromes P-450 were shown to exist in the inner membrane of adrenocortical mitochondria. These results are consistent with previously reported results of Mitani *et al.*[24], who used polyclonal antibodies raised against these cytochromes P-450.

Acknowledgements—We are grateful to Drs M. Takahashi, K. Yamanishi and T. Okuno of the Microbial Disease Research Institute of Osaka University for their valuable advice during the preparation of hybridomas. The authors also thank Drs T. Sugiyama and Y. Nonaka of our department for their interest and kind advice during this study. This work was supported in part by research grants from the Science and Technology Agency of Japan, the Ministry of Health and Welfare of Japan, the Ministry of Education, Science and Culture of Japan, Research Foundation for Cancer and Cardiovascular Diseases, and Chiyoda Mutual Life Foundation.

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