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Monoclonal Anti-Tyrosine Hydroxylase Antibodies (41239)

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Abstract. Tyrosine hydroxylase (TH) and cytosol fractions from human mammary carcinoma and malignant melanoma cells bind 17β-[^{3}H]estradiol with strong affinity. Sucrose density gradients suggested differences between the binding of 17β-[^{3}H]estradiol to TH and melanoma cell cytosol compared with the binding of the estrogen to mammary carcinoma. Monoclonal antibodies secreted by cloned cell hybrids which were formed by the fusion of murine myeloma cells with spleen cells from BALB/c mice immunized with purified TH inhibited the enzymatic activity and the binding of 17β-[^{3}H]estradiol to TH or to cytosol fractions of melanotic melanoma cells, but had no effect on 17β-[^{3}H]estradiol binding to cytosol fractions from various mammary epithelial or carcinoma cell lines. Cultivation of estradiol-responsive melanotic melanoma cells in media supplemented with the TH antibodies produced loss of pigmentation, and the cells became amelanotic and nonresponsive to 17β-estradiol. The results indicate that tyrosine hydroxylase of melanotic melanoma cells is an estrogen-binding protein.

Malignant melanomas have been claimed to have cytoplasmic estrogen receptors as determined by means of Scatchard analysis of estrogen-binding data and a specific relationship has been suggested between estrogen and melanoma (1, 2). Therefore the specific question which led to the present investigation was the relation between estradiol-binding proteins in cytosol fractions of *in vitro* cultured human malignant melanoma cells to the estradiol receptor (ER) in mammary carcinoma cells.

Tyrosine hydroxylase (TH) is a key enzyme in melanogenesis. Using disc electrophoresis and melanoma tissue, the enzyme was shown to exist in multiple molecular forms (3). In mouse and human tumors the T_1 and T_2 tyrosine hydroxylases are soluble, while T_3 is a particulate insoluble enzyme (4–7).

Melanin, a unifying pigment differentiating melanomas from other tumors (8), arises from the oxidation of tyrosine. Melanin biosynthesis is a specific differentiated function of melanocytes, and tyrosine hydroxylase is a specific enzyme for melanogenesis in melanotic melanoma cells.

When examined for estrogen receptors, all tumors which bound 17β-[^{3}H]estradiol were melanin producing (melanotic). Analysis of the apparent estrogen binding by sucrose density gradients indicated no distinct 4 S, 6 S, or 8 S peaks which characterized estrogen-receptor binding (9), suggesting the presence in melanoma cells a new estrogen-binding site.

Several investigators have tried to produce monoclonal antibodies against various human tumor antigens or markers (10), including melanoma (11, 12), neuroblastoma (13), colon carcinoma (14, 15), and carcinoembryonic antigen (16). Monoclonal antibodies secreted by hybrids obtained from fusion of murine myeloma cells (17) and spleen cells from mice immunized with a membrane-enriched fraction of melanoma cells had a restricted reactivity toward melanoma cells as assessed by indirect antibody-binding radioimmunoassay (18, 19). The antibodies secreted by two hybrids were directed against two different antigenic determinants expressed on 15 of the 16 different melanoma cell lines tested (20).

The present studies describe the action of supernatants from cultures of cloned cell

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hybrids obtained from fusion of murine myeloma cells with spleen cells of BALB/c mice immunized with purified tyrosine hydroxylase. These supernatants inhibited the enzyme catalytic activity, and the binding of 17β-[^{3}H]estradiol to tyrosine hydroxylase. These TH antibodies inhibited 17β-[^{3}H]estradiol binding to cytosol fractions from melanotic melanoma cells. They had no effect on either mammary carcinoma or normal mammary epithelial cells. The TH antibodies converted melanotic melanoma to amelanotic cells.

Materials and Methods. Purified tyrosine hydroxylase (polyphenoloxidase, Agatius) was obtained from Sigma Chemical Company, 4000 U/mg and further purified to give one single band on polyacrylamide gel electrophoresis.

Cell lines. a. Human skin fibroblast (HSF), a diploid cell line, was established from human skin biopsies as explant from adults without tumor (21, 22). The fibroblast cell line was on its 25th passage, and it was checked every 2 months for the production of the fibroblast growth-promoting factor.

b. Human normal mammary epithelial cells were grown on gelled collagen (23). A basal medium consisting of Hams F12 (GIBCO) supplemented with 10% horse serum, 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), and cholera toxin (0.01 μg/ml) was used to grow the normal epithelial cells. A solution of 1% collagenase made up in Hanks' basal salt solution (HBSS) was used to recover the cells.

c. Human malignant melanoma cell lines HMMC-ShA and HMMC-WJP were established using tumor biopsies as explants from two male adults, whereas HMMC-ZBJ cell line was from female adult, histologically proven as malignant melanoma (24). In the present studies the cell lines HMMC-ShA, HMMC-WJP, and HMMC-ZBJ were in their 151st, 125th, and 105th passages, respectively, and were examined at 60-day intervals. All tests for mycoplasma (25, 26) were negative. The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (GIBCO) supplemented with 10% (v/v) heat-inactivated (30 min, 56°) fetal calf serum (FCS) and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; and gentamicin, 50 μg/ml) in tissue culture dishes (Falcon Plastics, Oxnard, Calif.). Cells were incubated at 37° in a 5% CO_2 humidified air atmosphere. For passage and at the termination of each experiment, the cells were detached with 2 m*M* EDTA in phosphate-buffered NaCl solution. Cells were counted and viability was determined from the proportion of cells excluding 0.1% trypan blue.

d. Human mammary carcinoma cell lines HMCC-SE, HMCC-BS, HMCC-GM, and HMCC-KM were at their 26th, 65th, 85th, 110th, and 116th passage, and were described in detail in earlier reports (27, 28).

Immunization. Four groups, 10 animals per group, of BALB/c (10–20 g) female mice were immunized with purified tyrosine hydroxylase, cytosol fractions from cell lines HMMC-ShA and HMCC-GM, and human albumin (control). A series of four intradermal injections of the agent, at multiple sites on the back, were administered during a 4-week period. The primary injection, 1.0 μg of the purified enzyme, or 1.0 μg of each cytosol protein, was given as an emulsion in Freund's complete adjuvant in the right thigh. Booster injections (1.0 μg of the enzyme or of the cytosol protein) were given in incomplete adjuvant.

Forty-eight hours prior to splenectomy, the mice received an intravenous (through the tail vein) injection of the immunizing agent. While anethesized and prior to splenectomy, blood was drawn from each animal and sera prepared. The sera of each group were pooled, adsorbed with human fibroblasts (1.0 ml serum per 10^7 cells) three times, then assayed by immunoprecipitation.

The spleens were removed, and pooled into their respective groups, then teased through sterile sieve mesh 80. Spleenocyte suspensions were prepared and splenic lymphocytes were separated on Ficoll–Hypaque columns.

Cell fusion. An equal number of 10^6 of

spleen lymphocytes and myeloma (NS1/1) cells were mixed in DMEM and centrifuged at 600*g* for 10 min. The supernatant was removed, and the cell pellets were carefully suspended in 1.5 ml of 50% polyethylene glycol 1500 in DMEM (pH 7.5–7.8) (29). After the cells were washed with two 3-ml portions of DMEM, they were incubated overnight in DMEM containing 20% FCS and antibiotics, then dispersed in DMEM containing hypoxanthine (0.1 m*M*), amthopterin (0.4 μM), and thymidine (3 μM) (HAT) and 20% FCS. Aliquots of each suspension were incubated at 37° in 96-well Coster plates in a humidified 5% CO_2–air atmosphere. The wells were fed 1 week later with 100 μl of HAT-free DMEM supplemented with 20% FCS. Wells that became acidic 7–14 days after fusion and contained visible cell clusters were assayed for anti-tyrosine hydroxylase by the double-antibody precipitation technique.

Assay for tyrosine hydroxylase (EC 1.10.3.1). The tyrosine hydroxylase activity in living cells and cytosol fractions was determined from the amount of ^3H_2O released into the medium during conversion of 1-[*ring*-3′,5′-^{3}H]tyrosine to dihydroxyphenylalanine according to the well-established procedures of Oikawa *et al.* (30) and Pomerantz (31).

Determination of melanin. Melanin content was measured by the colorimetric method described by Whittaker (32). Briefly, in duplicate Beckman microfuge polyethylene tubes 5×10^6 cells were suspended, lysed in 0.5 ml of deionized H_2O, and subjected to cycles of freezing and thawing. Perchloric acid was added to final concentration of 0.5 *N* and the suspension was kept on ice for 10 min, then centrifuged for 5 min. After extraction with 0.5 *N* $HC10_4$ and two extractions with cold ethyl alcohol–ethyl ether (2:1 v/v) and ether, pellets were dissolved in 1.0 ml of 0.55 *N* KOH at 100° for 10 min. After centrifugation the supernatant was cooled to room temperature, and absorbance at 400 nm was measured in a Beckman DU 2 spectrophotometer. A standard curve was constructed using synthetic melanin (Sigma) dissolved in hot KOH at concentrations between 1 and 150 μg/ml, which gave a straight line at 400 nm.

[^{3}H]Estradiol binding to tumor cell cytosols and to purified tyrosine hydroxylase. Estrogen-binding protein content of tumor cells was determined using standard methods (33, 34). Briefly, 1.5 ml (10^6 cells/ml) in Tris: EDTA buffer (10 m*M* Tris–HCl:1.5 m*M* EDTA:1 m*M* dithiothreitol:10% glycerol at pH 7.4) was exposed to three cycles of quick freezing–thawing, then centrifuged at 100,000*g* for 45 min. The supernatant protein content was analyzed by means of the Coomassie dye method. In triplicates, aliquots of 200 μl containing 100 μl of [^{3}H]estradiol (0.05 μCi, 90 Ci/mmole; final concentration, 0.70×10^{-9} *M*), 20 μl of nafoxidine (final concentration, 1.6×10^{-9} *M*), or 20 μl of the antibodies and 300 μl of buffer were incubated for 60 min at 4°. One hundred microliters of 1.5% dextran-coated charcoal in Tris:EDTA was added, set for 30 min, and then centrifuged. Supernatants were removed, and radioactivity was determined.

To determine the specific binding protein, aliquots of 20 μl of (5×10^{-6} *M*) nafoxidine (200:1) or the monoclonal antibodies or buffer alone was added to 200-μl samples of tumor cytosol, after 30 min at 4°, 100 μl of [^{3}H]estradiol (0.05 μCi, final concentration) was added and incubated at 4° for an additional 90 min. Unbound labeled estradiol was removed by addition of 100 μl of 1.5% solution of dextran-coated charcoal, then centrifuged at 5000*g*. Two hundred microliters of the supernatants was layered on a linear 10 to 30% sucrose gradient in Tris buffer and centrifuged at 45,000 rpm for 15 hr in SW-60 Beckman rotor at 4°. Gradients were fractionated by means upward displacement with 75% glycerol into 40 fractions. Radioactivity was measured by liquid scintillation counting in a Packard Tri-Carb Model 3330 with a counting efficiency of 55%. Both ^{14}C-labeled bovine serum albumin (4.5 S) and gamma globulin (7 S) were used as molecular weight standards.

The effect of tamoxifen and monoclonal antibodies. To determine the effect of

tamoxifen and monoclonal antibodies on estradiol-binding receptor, tamoxifen (1.25 mM) or monoclonal antibodies (1 μg protein) were mixed with the cytosol fractions, [^{3}H]estradiol (0.25 nM) with increasing concentrations of cold estradiol were added, and the mixtures were incubated for 16 hr at 4°. Protein-bound and unbound ligands were separated by centrifugation at 1500g for 10 min in the presence of dextran-coated charcoal. Radioactivity in aliquots of the supernatants was then counted.

Results. *Characteristics of human normal mammary epithelial cells.* Human normal mammary epithelial cells produced duct-like outgrowth extending into the collagen gel matrix within 4 days after cultivation in the presence of cholera toxin and horse serum (Fig. 1). The toxin was included in the culture medium to inhibit the growth of fibroblastic cells (35) and to stimulate epithelial cell proliferation (36). When plated at high density (1 × 10^6 cells/cm^2) in conventional monolayer cultures in presence of cortisol at 10 μg/ml, the cells showed dome formation (37). Electron microscopy of the growing cells showed polarized cells containing many desmosomes, microvilli, and cytoplasmic blebs with tight junctions (Fig. 2). These are well-established characteristics of mammary epithelial cells ((38), manuscript under preparation).

[^{3}H]Estradiol binding to purified tyrosine hydroxylase and cytosol fractions of human malignant melanoma and mammary carcinoma cells. Scatchard analysis of [^{3}H]-estradiol binding to cytosol from mammary carcinoma, melanoma cells, and a system with 10 μg of purified tyrosine hydroxylase (TH) is presented in Fig. 3. These analyses were completed by means of multiconcentration saturation analysis using the dextran-coated charcoal analysis (DCCA) of four mammary carcinoma and three malignant melanoma cell lines. Apparent estrogen binding of more than 5 fmole/mg protein was observed in all the tumor cell cytosols.

Curve A in Fig. 3, is the plot for [^{3}H]-estradiol binding to tyrosine hydroxylase. The plot shows high affinity and resembles those for melanoma cell cytosols (curve B). Curve B is a representative plot for [^{3}H]-estradiol binding to cytosols of malignant melanoma cell lines. The binding was of high affinity with K_a between 3.1 × 10^{-9} and 1.7 × 10^{-10} M. Curve C is a representative plot for [^{3}H]estradiol binding to cytosols of mammary carcinoma cell lines. The data demonstrate one single class of binding component with K_d = 3.9 × 10^{-10} M and n = 83.8 fmole/mg protein.

Lineweaver–Burk plots for l-[ring-3′,5′-^{3}H]tyrosine hydrolysis by tyrosine hydroxylase in presence and absence of monoclonal TH antibodies. Figure 4 shows a double reciprocal Linweaver–Burk plot for the cleavage of 1-[*ring*-3′,5′-^{3}H]tyrosine by purified tyrosine hydroxylase in the presence and absence of monoclonal TH antibodies. The enzyme assayed without the antibody, was found to have a K_m (app) value of 0.05 mM and a V_{max} of 2.18 μM min^{-1} at 23° and pH 7.5 (0.53 μg enzyme/ml). When monoclonal TH antibodies were present, the double reciprocal plot gave the same V_{max} but different K_m (app) suggesting that the antibodies were acting as competitive inhibitors of [^{3}H]tyrosine hydrolysis. The K_i of the monoclonal TH antibody as an inhibitor was found to be 0.1 ng of protein.

Figure 4B shows a double reciprocal plot for the cleavage of [^{3}H]tyrosine by tyrosine hydroxylase from melanoma cell cytosol in presence and absence of the TH antibodies. The melanoma–cytosol tyrosine hydroxylase assayed without monoclonal TH antibody was found to have a K_m (app) of 0.11 mM and a V_{max} of 1.65 μM min-1 (corrected to correspond to the same amount of the purified enzyme used in Fig. 4A). When the monoclonal TH antibody was present, the double reciprocal plot gave the same V_{max} but different K_m (app′), suggesting that the antibody was acting as competitive inhibitor of [^{3}H]tyrosine hydrolysis.

Effect of monoclonal TH antibodies on [^{3}H]estradiol binding, tyrosine hydroxylase activity, and melanin biosynthesis. The data in Table I summarize the effect of monoclonal TH antibodies on 17β-estradiol binding and tyrosine hydroxylase activity of cytosols from various *in vitro*-cultivated

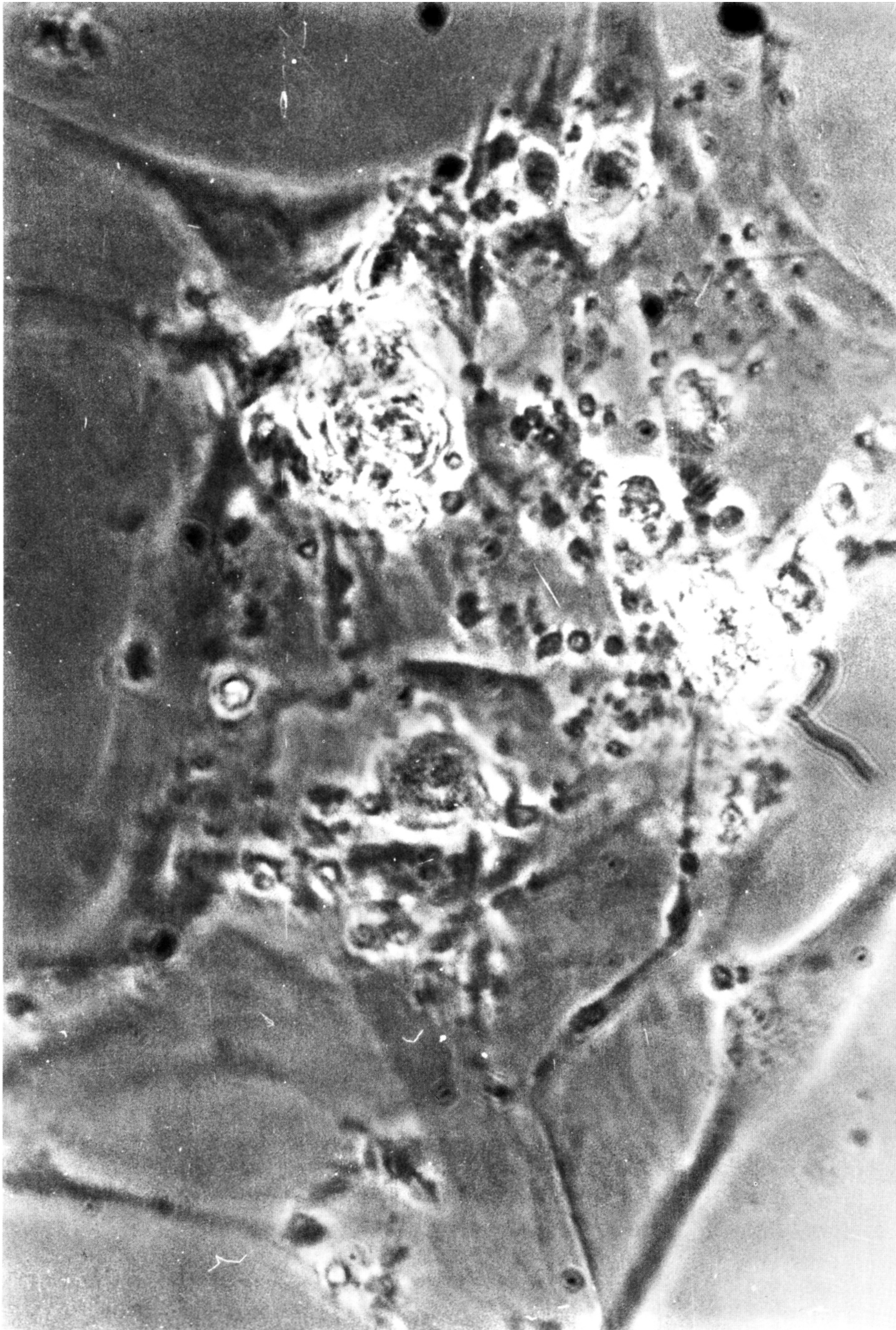

FIG. 1. Phase-contrast photomicrograph of human normal mammary epithelial cells (10^5 cells) cultured for 6 days in Hams F12 medium containing 10% horse serum, 5% fetal calf serum, 0.01 μg of cholera toxin per milliliter, and antibiotics as described in text (phase ×80).

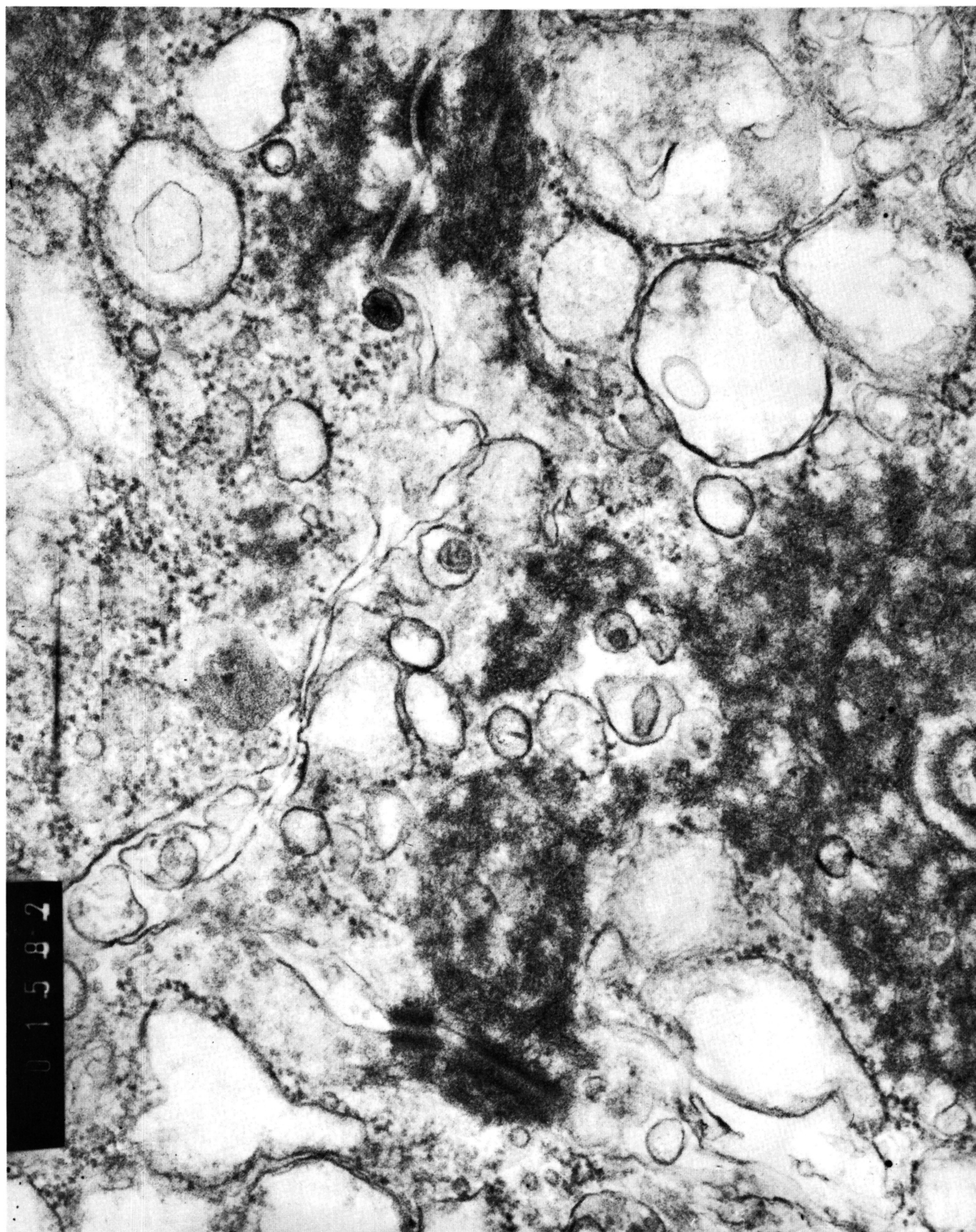

FIG. 2. Electron micrographs of human normal mammary epithelial cells cultured for 6 days in Hams F12 medium containing 10% horse serum, 5% fetal calf serum, 0.01 μg cholera toxin per milliliter, and antibiotics as described in text. Magnification ×28,000.

human cells. 17β-Estradiol binds to tumor cell cytosols at varying magnitudes, which varied between 447 ± 9 to 1385 and 578 to 848 fmole/mg protein for mammary carcinoma and melanoma cells, respectively. Also, estradiol strongly binds to tyrosine hydroxylase and to a weaker extent to albumin. The data indicate that monoclonal

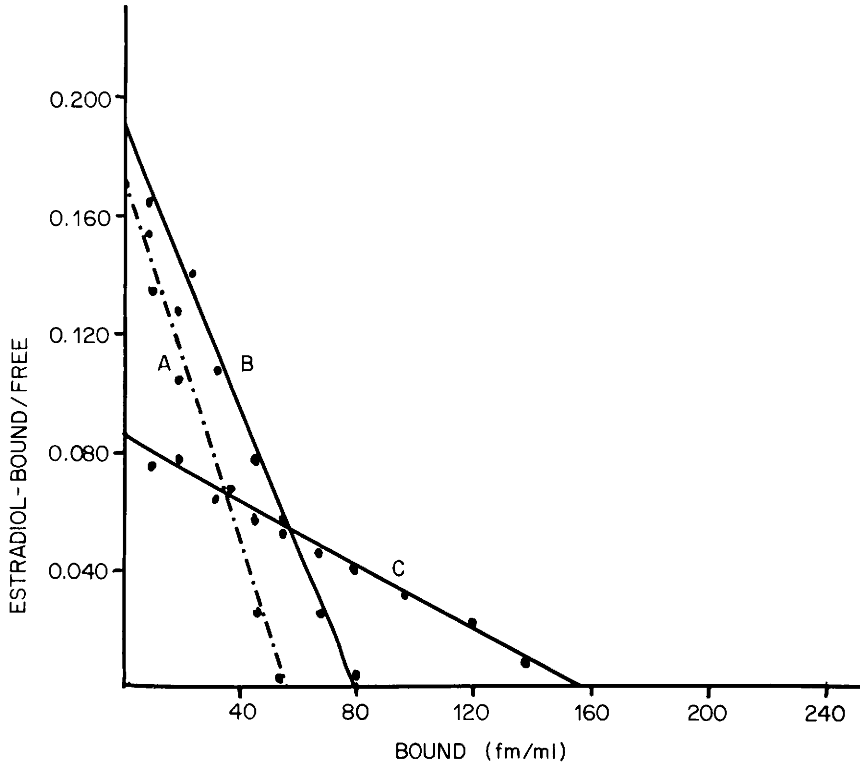

FIG. 3. Scatchard plot of [^{3}H]estradiol binding: (A) in a system with 10 μg of purified tyrosine hydroxylase, and in cytosols of (B) human malignant melanoma and (C) human mammary carcinoma cells. Experimental details are described in text.

TH antibodies inhibited [^{3}H]estradiol binding to tyrosine hydroxylase and to cytosols of melanoma cells, but had no effect on the binding to cytosols of mammary carcinoma or of normal epithelial cells.

Tyrosine hydroxylase activity varied from one melanoma cell line to another, i.e., from 954 units in melanotic and to 9 units in amelanotic melanoma cells. Mammary carcinoma cells had lower activities, i.e., 12 to 51 units. The monoclonal TH antibodies inhibited tyrosine hydroxylase activities of the melanotic melanoma cells.

Melanin synthesis is a specialized function of pigment cells and differentiates melanotic melanoma cells from other tumor cells. Melanin production varied from one melanoma cell line to another (0.21 to 1.31 A_{400} units/10^6 cells). Skin fibroblasts also synthesized melanin, but to a much lower extent. Mammary carcinoma cells and normal mammary epithelial cells lack the capacity to synthesize melanin. Monoclonal TH antibodies inhibited melanin synthesis in melanotic melanoma cells.

Effect of tamoxifen and L-DOPA on estradiol binding and tyrosine hydroxylase activity. The data in Table II, summarize the effect of tamoxifen on 17β-[^{3}H]estradiol binding and tyrosine hydroxylase activity of cytosols from various *in vitro*-cultured human cells. Tamoxifen had statistically nonsignificant effect on [^{3}H]estradiol binding and tyrosine hydroxylase activity of cytosols from malignant melanoma cells and on purified tyrosine hydroxylase. On cytosols from human mammary carcinoma and epithelial cells, tamoxifen caused 73 to 85 and 94 to 98% inhibition of [^{3}H]estradiol binding, respectively.

Addition of L-DOPA to incubation mixtures containing tyrosine hydroxylase produced 79 and 82% inhibition of the observed binding of estradiol and enzymatic

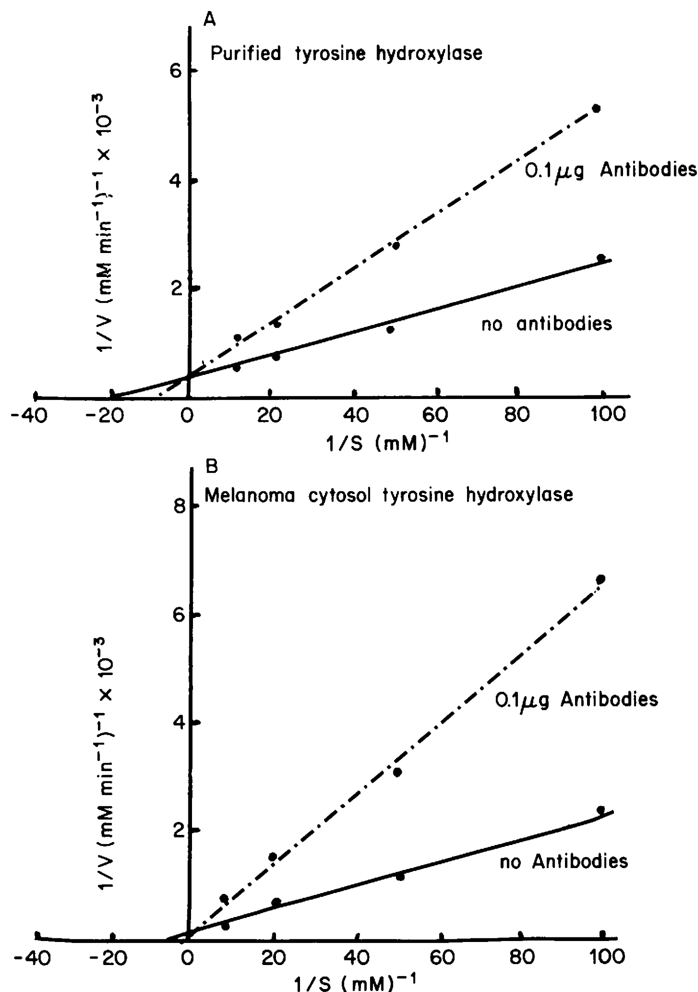

FIG. 4. Lineweaver–Burk plots for l-[*ring* 3′,5′-^{3}H]tyrosine in the presence and absence of monoclonal TH antibodies. (A) Purified tyrosine hydroxylase (40 μg): [^{3}H]tyrosine without monoclonal antibodies (——) and with 0.1 μg monoclonal TH antibodies (– · — · –). (B) Tyrosine hydroxylase from cytosol of malignant melanoma cells (40 μg protein): [^{3}H]tyrosine without monoclonal TH-antibodies (——), and with 0.1 μg monoclonal TH-antibodies (– · — · –). The experiments were performed as described in text.

activity, respectively. L-DOPA caused 76 to 89 and 78 to 92% inhibition of the observed binding of estradiol and tyrosine hydroxylase activities of three melanotic melanoma cell lines. L-DOPA had no apparent effect on estradiol binding to amelanotic melanoma or to mammary carcinoma cell cytosols (Table II).

Effect of monoclonal antibodies from various cloned cell hybrids on [^{3}H]estradiol binding to cytosols of human cell lines. The data in Table III, summarize the effect of various monoclonal antibodies secreted by certain cloned cell hybrids on [^{3}H]estradiol binding to cytosols of human cells.

Two cloned cell hybrids Hy-7 and Hy-7′, which were produced from fusion of murine myeloma cells with spleen lymphocytes of animals preimmunized with tyrosine hydroxylase and with cytosols of melanoma cells, inhibited [^{3}H]estradiol binding to tyrosine hydroxylase and to cytosols of

TABLE I. EFFECT OF MONOCLONAL TYROSINE HYDROXYLASE ANTIBODIES

Binding ligand	[^{3}H]Estradiol binding (fmole/mg protein)		Tyrosine hydroxylase (^3H_2O formed cpm/hr/10^6 cells)		Melanin synthesis ($A_{400}/10^6$ cells)	
	Standard	+ *M*-THA	Standard	+ *M*-THA	Standard	+ *M*-THA
Human albumin	182 ± 12	171 ± 12	—	—	—	—
Tyrosine hydroxylase	963 ± 62	8.5 ± 0.2	1065 ± 75	0.9 ± 0.01	—	—
Cytosol fraction from						
HMMC-ShA	624 ± 37	74 ± 3.2	485 ± 32	0.8 ± 0.01	1.300 ± 0.01	0.190 ± 0.01
HMMC-WJP	848 ± 64	67 ± 5.4	954 ± 63	0.9 ± 0.01	1.664 ± 0.04	0.213 ± 0.01
HMMC-ZBJ	578 ± 42	53 ± 4.2	345 ± 29	0.4 ± 0.01	0.935 ± 0.05	0.115 ± 0.02
HMMC-SR	20 ± 1.8	1.8 ± 0.1	9 ± 0.6	0.1 ± 0.01	0.213 ± 0.01	0.007 ± 0.001
HMCC-GM	447 ± 29	426 ± 18	12 ± 0.8	0.4 ± 0.01	0.062 ± 0.01	0.073 ± 0.02
HMCC-BS	1127 ± 75	1130 ± 76	47 ± 3.2	0.5 ± 0.02	0.085 ± 0.01	0.094 ± 0.01
HMCC-KM	975 ± 58	982 ± 61	38 ± 1.9	0.4 ± 0.01	0.075 ± 0.01	0.053 ± 0.004
HMCC-SE	1385 ± 81	1381 ± 80	51 ± 3.7	0.5 ± 0.01	0.097 ± 0.01	0.062 ± 0.007
HNMEC-1	25 ± 2.5	17 ± 1.8	11 ± 0.7	0.1 ± 0.01	0.04 ± 0.01	0.017 ± 0.001
HNMEC-2	19 ± 1.8	8 ± 1.2	8 ± 0.6	0.1 ± 0.01	0.012 ± 0.01	0.011 ± 0.001
HNMEC-3	37 ± 2.9	16 ± 1.8	14 ± 2.6	0.1 ± 0.03	0.054 ± 0.01	0.010 ± 0.001
HSF	18 ± 2.8	2.5 ± 0.1	16 ± 1.7	0.1 ± 0.01	0.326 ± 0.03	0.087 ± 0.003

Note. HMMC-, HMCC-, HNMEC-, and HSF represent human malignant melanoma, human mammary carcinoma, human normal epithelial, and human fibroblasts, respectively. The above data represent the average ± SD of three experiments, each run in duplicates.

TABLE II. Effect of Tamoxifen and L-DOPA on Estradiol Binding and Tyrosine Hydroxylase (Percentage Inhibition)

Binding ligand	Tamoxifen		L-DOPA	
	Estradiol binding[a]	Tyrosine hydroxylase[b]	Estradiol binding	Tyrosine hydroxylase
Tyrosine hydroxylase	2.5 ± 0.1	1.9 ± 0.1	79 ± 6	82 ± 7
Cytosol fraction from				
HMMC-ShA	4.7 ± 0.2	8.9 ± 0.7	85 ± 7	87 ± 8
HMMC-WJP	3.5 ± 0.2	3.1 ± 0.2	76 ± 6	78 ± 6
HMMC-ZBJ	6.8 ± 0.3	6.2 ± 0.2	89 ± 9	92 ± 9
HMMC-SR	1.1 ± 0.1	1.2 ± 0.2	2.1 ± 0.1	2.4 ± 0.2
HMCC-GM	85 ± 7.2	1.3 ± 0.1	8.7 ± 0.8	ND
HMCC-BS	79 ± 6.9	6.9 ± 0.1	6.8 ± 0.4	ND
HMCC-KM	81 ± 7.0	1.7 ± 0.1	6.5 ± 0.3	ND
HMCC-SE	79 ± 6.0	1.1 ± 0.1	6.9 ± 0.3	ND
HNMEC-1	97 ± 8.8	ND	12.6 ± 1.1	ND
HNMEC-2	94 ± 8.7	ND	9.8 ± 0.9	ND
HNMEC-3	98 ± 9.1	ND	15.7 ± 1.3	ND
HSF	2.1 ± 0.1	8.7 ± 0.1	76 ± 6.8	82 ± 7.3

Note. HMMC, HMCC-, HNMEC-, and HSF indicate malignant melanoma, mammary carcinoma, normal mammary epithelial, and skin fibroblasts, respectively.
[a] Monitored by fmole bound/mg protein.
[b] Monitored by ^3H_2O formed cpm/hr/10^6 cells.
[c] The data are presented as percentage inhibition ± SD of three separate experiments. Each was carried out in duplicates.

melanoma cells. The two antibodies had no effect of the estrogen binding to cytosols of normal mammary epithelial or mammary carcinoma cells. The antibodies secreted by Hy-24 (a cloned cell hybrid from fusion of the murine myeloma cells with spleen lymphocytes of animals preimmunized with cytosols of melanoma cells) inhibited estrogen binding and tyrosine hydroxylase activities of the immunizing cells, i.e., HMMC-ShA cells.

Fusion of the murine myeloma cells with spleen lymphocytes from animals immunized with cytosols of mammary carcinoma HMCC-BS cells formed three cloned cell hybrids (Hy-9, Hy-32, and Hy-58) that produced antibodies. Antibodies from Hy-9 inhibited estradiol binding to cytosols of the immunizing cells, i.e., HMCC-BS. The antibodies from Hy-32 strongly inhibited and reduced the estrogen binding to cytosols of the four mammary carcinoma cell lines and of normal mammary epithelial, respectively. Antibodies from Hy-58 reduced equally estrogen binding to cytosols of both normal mammary epithelial and mammary carcinoma cells. Therefore, these experiments showed the following:

1. Monoclonal tyrosine hydroxylase antibodies inhibited estradiol binding to cytosols from various melanotic melanoma cell lines, but had no effect on the estradiol binding to cytosols of mammary carcinoma cells.
2. In malignant melanotic melanoma cells, there are at least two antigenic determinants, one specific to the cell cytosol immunizing the animal, and the other common to the four melanoma cell lines. Neither antibodies had any effect on the estradiol binding to cytosols of mammary carcinoma. Both antibodies inhibited estrogen binding to purified tyrosine hydroxylase.
3. In cytosols of the mammary carcinoma, there are at least three antigenic determinants, one specific to the immunizing cell, the second common to the four mammary carcinoma cell lines, and the third cross-reacted with normal mammary epithelial cells.

Effect of L-DOPA and monoclonal TH antibodies on melanotic melanoma cell

TABLE III. EFFECT OF VARIOUS MONOCLONAL ANTIBODIES ON [^{3}H]ESTRADIOL BINDING

Binding ligand	Monoclonal antibodies from cloned hybrids					
	Hy-7	Hy-7′	Hy-24	Hy-9	Hy-32	Hy-58
Human albumin	2.7 ± 0.1	2.5 ± 0.1	14 ± 0.8	11 ± 1.5	8.4 ± 0.7	9.3 ± 0.8
Tyrosine hydroxylase	98 ± 6	96 ± 7	35 ± 4	4.5 ± 0.1	2.9 ± 0.1	5.6 ± 0.2
Cytosol fraction from						
HMMC-ShA	89 ± 5	94 ± 8	93 ± 8	3.7 ± 0.1	6.5 ± 0.4	9.2 ± 0.8
HMMC-WJP	92 ± 6	96 ± 9	15 ± 3	5.4 ± 0.3	7.4 ± 0.8	8.3 ± 0.9
HMMC-ZBJ	87 ± 6	92 ± 8	12 ± 2	9.2 ± 0.8	4.6 ± 0.2	7.9 ± 0.5
HMMC-SR	82 ± 7	91 ± 7	8.6 ± 0.9	7.5 ± 0.6	3.1 ± 0.2	2.8 ± 0.1
HMCC-GM	4.7 ± 0.2	3.4 ± 0.1	6.9 ± 0.5	17 ± 2.5	89 ± 7.3	37 ± 2.7
HMCC-BS	11 ± 0.7	8.1 ± 0.3	10 ± 0.6	98 ± 8.3	96 ± 8.2	29 ± 3.1
HMCC-KM	6.9 ± 0.3	4.7 ± 0.2	7.5 ± 0.5	11 ± 0.9	94 ± 9.2	34 ± 2.8
HMCC-SE	9.3 ± 0.8	5.1 ± 0.2	9.3 ± 0.4	9.6 ± 0.8	92 ± 9.1	19 ± 2.0
HNMEC-1	3.6 ± 0.1	2.5 ± 0.1	3.5 ± 0.1	12 ± 0.9	9.4 ± 0.4	25 ± 0.1
HNMEC-2	4.8 ± 0.1	5.7 ± 0.1	8.3 ± 0.4	10 ± 0.7	7.4 ± 0.3	29 ± 2.4
HNMEC-3	6.7 ± 0.1	7.1 ± 0.3	11 ± 0.9	8.6 ± 0.6	6.4 ± 0.4	17 ± 0.8
HSF	78 ± 6.3	85 ± 7.2	13 ± 0.9	7.3 ± 0.3	5.8 ± 0.1	9.6 ± 0.8

Note. The cloned hybrids were produced from fusion of mouse myeloma cells with spleen lymphocytes of animals preimmunized with: tyrosine hydroxylase (Hy-7), with HMMC-ShA (Hy-7′, Hy-24), and with HMCC-BS (Hy-9, Hy-32, Hy-58). The effect was monitored by the binding of [^{3}H]estradiol. The data are presented as percentage inhibition ± SD of three separate experiments. Each was carried out in duplicates.

lines. Parallel cultures of melanotic melanoma cell lines (HMMC-ShA, HMMC-WJP, and HMMC-ZBJ) were cultivated in DMEM supplemented with fetal calf serum and with either monoclonal TH antibodies (0.5 μg protein/ml) or L-DOPA (10 μg/ml). At 72-hr intervals, the time of feeding, aliquots of each culture were examined for estradiol binding, tyrosine hydroxylase activity, and melanin production. The data summarized in Table 4 indicate that cultivation of melanotic melanoma cells in culture media supplemented with either monoclonal TH antibodies or L-DOPA, decreased (18 and 32%) [^{3}H]estradiol binding to the cytosols. If the cells were carried on in such culture media, the ability to bind estradiol decreased progressively to 9–27 and 8–10 fmole/mg protein, respectively. Simultaneous decrease occurred in cytosol tyrosine hydroxylase activities, and melanin biosynthesis. These changes did not occur in control cultures carried on in parallel in the standard culture medium. This transformation of melanotic to amelanotic state induced by monoclonal tyrosine hydroxylase antibodies and by L-DOPA constitute the second major finding of the described studies. Aside from the possibility that continuous inhibition of tyrosine hydroxylase could suppress the synthesis of both the enzyme and melanin, the mechanisms of this transformation are under current investigation.

Immunoprecipitation reaction with antisera and monoclonal antibodies. Table V summarizes the highest dilution of the antiserum or of the monoclonal antibody which formed precipitate with the immunizing agent. It indicates:

1. Human albumin formed precipitin when reacted with 1:64 dilution of serum from animals immunized with albumin, and with 1:2 dilution of sera from animals immunized with tyrosine hydroxylase, and with cytosols of HMMC-ShA or HMcc-GM cells. But, when reacted with monoclonal antibodies, albumin formed no precipitin.

2. When reacted with purified tyrosine hydroxylase, sera from animals immunized with tyrosine hydroxylase and cytosols of HMMC-ShA or HMCC-GM formed precipitin at 1:128, 1:64, and 1:4 dilutions, whereas, monoclonal antibodies from Hy-7, Hy-7′, Hy-24, and Hy-9 formed precipitin at 1:256, 1:128, 1:4, and 1:4 dilutions, respectively.

3. When reacted with cytosols of mela-

TABLE IV. EFFECT OF MONOCLONAL TYROSINE HYDROXYLASE ANTIBODIES AND L-DOPA ON MELANOTIC MELANOMA CELLS

Cell lines	Cultivation period (hr)	[^{3}H]Estradiol binding (fmole/mg protein)			Tyrosine hydroxylase (^3H_2O formed cpm/hr/10^6 cells)			Melanin synthesis ($A_{400}/10^6$ cells)		
		Standard	+ *M*-THA	+ L-DOPA	Standard	+ *M*-THA	+ L-DOPA	Standard	+ *M*-THA	+ L-DOPA
HMMC-ShA	72	595 ± 35	487 ± 29	372 ± 19	445 ± 30	327 ± 21	245 ± 17	1.30 ± 0.01	0.92 ± 0.01	0.78 ± 0.01
HMMC-WJP	72	817 ± 45	658 ± 40	524 ± 32	807 ± 68	727 ± 52	645 ± 45	1.66 ± 0.04	1.32 ± 0.03	1.05 ± 0.01
HMMC-ZBJ	72	548 ± 29	442 ± 25	327 ± 17	425 ± 78	325 ± 27	217 ± 14	0.93 ± 0.05	0.72 ± 0.02	0.59 ± 0.01
HMMC-ShA	144	373 ± 22	264 ± 19	173 ± 15	298 ± 19	185 ± 15	113 ± 12	1.04 ± 0.01	0.84 ± 0.02	0.65 ± 0.02
HMMC-WJP	144	587 ± 34	458 ± 31	347 ± 24	625 ± 39	517 ± 31	327 ± 19	1.37 ± 0.05	1.04 ± 0.03	0.75 ± 0.02
HMMC-ZBJ	144	357 ± 20	239 ± 18	122 ± 14	275 ± 22	198 ± 19	135 ± 11	0.69 ± 0.02	0.41 ± 0.02	0.15 ± 0.01
HMMC-ShA	216	147 ± 14	78 ± 6	37 ± 5	142 ± 14	79 ± 9	31 ± 4	0.76 ± 0.03	0.54 ± 0.02	0.42 ± 0.01
HMMC-WJP	216	238 ± 18	114 ± 9	58 ± 4	375 ± 34	187 ± 17	98 ± 8	0.85 ± 0.04	0.62 ± 0.03	0.53 ± 0.03
HMMC-ZBJ	216	114 ± 11	65 ± 7	31 ± 2	117 ± 11	84 ± 9	42 ± 4	0.32 ± 0.03	0.15 ± 0.02	0.10 ± 0.01
HMMC-ShA	288	19 ± 1.1	11 ± 0.8	9 ± 0.2	9.8 ± 0.8	4.7 ± 0.4	2.9 ± 0.2	0.15 ± 0.01	0.14 ± 0.01	0.09 ± 0.01
HMMC-WJP	288	32 ± 1.7	27 ± 1.3	16 ± 1.3	12 ± 0.9	9.3 ± 0.8	8.6 ± 0.6	0.21 ± 0.02	0.19 ± 0.01	0.11 ± 0.01
HMMC-ZBJ	288	14 ± 0.9	9 ± 0.8	8 ± 0.3	7.4 ± 0.6	4.8 ± 0.4	3.5 ± 0.3	0.11 ± 0.01	0.08 ± 0.01	0.07 ± 0.01

TABLE V. IMMUNOPRECIPITATION OF ANTISERA AND MONOCLONAL ANTIBODIES

Immunizing agents	Serum from mice immunized with				Monoclonal antibodies from cloned hybrids					
			Cytosols from cell lines							
	Human albumin	Tyrosine hydroxylase	HMMC-Sh.A	HMCC-GM	Hy-7	Hy-7′	Hy-24	Hy-9	Hy-32	Hy-58
Human albumin	1:64	1:2	1:2	1:2	—	—	—	—	—	—
Purified tyrosine hydroxylase	—	1:128	1:64	1:4	1:256	1:128	1:4	1:4	—	—
Cytosol from cell line										
HMMC-ShA	1:2	1:128	1:128	1:16	1:128	1:32	1:256	—	—	1:4
HMCC-GM	1:2	1:16	1:16	1:128	—	—	1:4	1:4	1:128	1:256

Note. The above data represent the highest dilution of the antibodies to produce precipitate with the immunizing agent. (—) indicates no evidence of precipitin formation at dilution of 1:2 ratio.

noma cells, sera from animals immunized with tyrosine hydroxylase and cytosols of HMMC-ShA or HMCC-GM formed precipitin at 1:128, 1:128, and 1:16 dilutions. Whereas monoclonal antibodies from Hy-7, Hy-7′, and Hy-24 formed precipitin at 1:128, 1:32, and 1:256 dilutions, respectively.

4. When reacted with cytosols of mammary carcinoma cells, sera from animals immunized with tyrosine hydroxylase and cytosols of HMMC-ShA or HMCC-GM formed precipitin at 1:16, 1:16, and 1:128 dilutions. Whereas, monoclonal antibodies from Hy-24, Hy-9, Hy-32, and Hy-58 formed precipitate at 1:4, 1:4, 1:128, and 1:256 dilutions, respectively. Therefore monoclonal antibodies from Hy-7 and Hy-24 formed precipitin with tyrosine hydroxylase and cytosol of melanoma cells HMMC-ShA, indicating immunological similarities.

Discussion. A major finding, obtained from the described experiments, was that using purified tyrosine hydroxylase as the immunogen, only 2 out of 40 cloned cell hybrids produced supernatants that inhibited (by competitive inhibition) the enzyme catalytic activity of the purified enzyme as well as that of cytosols from three melanotic melanoma cells. The results indicate that both enzymes have similar active center. Simultaneously, these same supernatants inhibited [^{3}H]estradiol binding to both the purified enzyme and the cytosols of melanotic melanoma cells, but had no effect on estradiol binding to cytosols of either mammary carcinoma or normal mammary epithelial cells. These results indicate the specificity of the antibody to tyrosine hydroxylase as a protein that binds estradiol, and not to other protein receptors for estradiol that are present in neoplastic and normal mammary epithelial cells. Further studies are in progress to define the relation between estrogen-binding site and the enzyme active center.

Hybridomas secreting anti-melanoma antibodies to cultured and freshly derived cells (35, 36), to cell surface antigens of human malignant melanoma (37), to rat mammary carcinoma (38), and to human estrogen receptor (39) have been produced in several laboratories. These monoclonal antibodies provided a basis for a simple immunoradiometric determination of estrogen receptors in human breast cancers and in identifying various antigens on the melanoma cell membrane.

There is close similarity between the binding of estradiol to purified tyrosine hydroxylase and to cytosols of melanotic melanoma. The use of monoclonal antibodies revealed differences in the kinetics of the two activities. The data suggested structural differences possibly attributed to the presence of isozymes, further studies are in progress.

Similar to monoclonal tyrosine hydroxylase antibodies, L-DOPA (L-β-3,4-dihydroxyphenylalanine), a specific inhibitor of tyrosine hydroxylase, inhibited estradiol binding to the purified enzyme and to cytosols of melanotic melanoma cells, with no effect on the binding of the estrogen to cytosols of either normal mammary epithelial or mammary carcinoma cells.

Using cytosols of mammary carcinoma as the immunogen, at least 3 out 40 cloned cell hybrid cells produced supernatants that inhibited [^{3}H]estradiol binding to cytosols of mammary carcinoma. One of these three supernatants inhibited [^{3}H]estradiol binding to cytosols of normal mammary epithelial cells, but none of these supernatants had any effect on the estrogen binding to cytosols of melanotic melanoma cells or to purified tyrosine hydroxylase. These active supernatants differed in their effects on cytosols of various mammary cell lines. The results indicated, at least in cytosols of human mammary carcinoma, there is a heterogeneous population of antigenic determinants, some of which are shared with cytosols from other mammary carcinoma and normal mammary epithelial cell lines.

Tamoxifen (1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene) is an estrogen antagonist. On the estrogen responsive human mammary carcinoma cell line, MCF-7, tamoxifen exerted its effects via the specific estrogen receptor proteins (39–41). Estradiol and tamoxifen are mutually competitive for the binding in DMBA-induced rat mammary carcinoma, and they bind to the same number of binding sites, i.e., to the classical cytoplasmic estrogen receptor sites (42). Tamoxifen inhib-

ited [^{3}H]estradiol binding to the estrogen receptor in a dose-dependent manner, but with an affinity considerably lower than that of estradiol (43–46). The present studies showed that tamoxifen had no effect on [^{3}H]estradiol binding to either cytosols of melanotic melanoma cells or to the purified tyrosine hydroxylase.

Tamoxifen and L-DOPA were used in parallel experiments. The results indicated that tamoxifen inhibited [^{3}H]estradiol binding to cytosols of human mammary carcinoma but not to cytosols of melanotic melanoma cells. Whereas, L-DOPA inhibited [^{3}H]estradiol binding to cytosols of melanotic melanoma, but not to cytosols of mammary carcinoma cells. These experiments showed that cytosols of mammary carcinoma and melanotic melanoma cells differed in their [^{3}H]estradiol-binding sites.

Using well-established standard techniques, the normal epithelial mammary epithelial cell cultures were free of mycoplasma and bacterial contamination. Density gradients were constructed with solutions of Ficoll, and were used to examine for contamination with fibroblasts. When mixtures of fibroblasts and epithelial cells (2×10^5) were centrifuged on the gradients for 10 min at 8160*g* only epithelial cells were recovered at the 1.065–1.085 g/ml interface zone. Whereas, the fibroblasts were recovered at the upper 1.025–1.050 g/ml zone. More than 98.7% of the normal mammary epithelial cell cultures were recovered at the epithelial cell zone (manuscript under preparation).

Aliquots of melanotic melanoma cells were cultured in parallel in (A) DMEM, (B) DMEM supplemented with monoclonal tyrosine hydroxylase, and (C) DMEM supplemented with L-DOPA. All the three cultures were incubated in the same incubator with 5% CO_2 humidified sterile air, fed at the same time and with the same volume of their nutrients, and they were split at the same time and at the same proportion. All the three types of cultures maintained approximately the same doubling time. The A culture remained melanotic, responsive to estradiol, and their cytosols maintained [^{3}H]estradiol-binding capacity and tyrosine hydroxylase activities. The B and C cultures became amelanotic and nonresponsive to estradiol and their cytosols did not bind [^{3}H]estradiol and had statistically nonsignificant tyrosine hydroxylase activities. Therefore, at least in human melanotic melanoma, there is a direct correlation between tyrosine hydroxylase, melanogenesis, i.e., differentiation, and estrogen responsiveness and binding capacities.

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