Antigenic Characterization of Human Hepatocellular Carcinoma

Development of In Vitro and In Vivo Immunoassays That Use Monoclonal Antibodies

Rolf I. Carlson, Edna Ben-Porath, Daniel Shouval, William Strauss, Kurt J. Isselbacher, and Jack R. Wands
Gastrointestinal Unit and Department of Nuclear Medicine, Massachusetts General Hospital, and Departments of Medicine and Radiology, Harvard Medical School, Boston, Massachusetts 02114; and The Liver Unit and Department of Medicine A, Hadassah University Hospital, Jerusalem 91120, Israel

Abstract

Several libraries of monoclonal antibodies have been produced by immunization of Balb/c mice with single cell suspensions of nontrypsin-treated human hepatocellular carcinoma cell (HCC) lines in order to study the antigenic properties of transformed hepatocytes. The antibodies were characterized with regards to specificity for hepatoma-associated antigens and their capability for use as reagents in radioimmunoassays (RIAs) and tumor localization in vivo. Three such antibodies namely, P215457, PM4E9917, P232524 of the IgG_{2a}, IgG_{2a}, and IgG_1 isotypes, respectively, not only recognized separate and distinct antigenic determinants on four human hepatoma cell lines but also reacted with epitopes present on chemically induced rat hepatoma cell lines. In contrast, only 1 of 38 other human malignant and transformed cell lines demonstrated reactivity with the three antibodies; normal human tissues were also found to be unreactive. Monoclonal antibody P215457 densely stained the plasma membrane by indirect immunofluorescence, showed rapid binding activity to HCC cells in suspension, and precipitated a 50,000-mol wt cell surface protein; antibody PM4E9917 also stained the plasma membrane and precipitated a 65,000-mol wt protein, whereas P232534 recognized cytoplasmic antigenic determinants. With these antibodies "simultaneous sandwich" RIAs were established that detect soluble hepatoma-associated antigens in culture supernatants. Finally, the Fab fragment of P215457 was found to be useful in tumor localization in vivo. This antibody fragment when labeled with ^{131}I was shown to localize by radionuclide-imaging studies in human hepatoma grown in nude mice. Thus, these investigations demonstrate that monoclonal antibodies may be produced against epitopes that reside almost exclusively on transformed hepatocytes and such antibodies may be successfully employed in the development of in vitro and in vivo immunoassays.

Introduction

Human hepatocellular carcinoma (HCC),[1] although unusual in industrialized western countries, occurs with such frequency

1. *Abbreviations used in this paper:* DTPA, diethylene triamine pentaacetic acid; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus;

in the populous countries of sub-Saharan Africa and the Far East that on a global basis it constitutes one of the most highly prevalent tumors (1). Unfortunately, because the onset of symptoms coincides with advanced stages of the disease, the prognosis is generally poor (2, 3). Once the tumor becomes widespread in the liver, surgical resection proves difficult and chemotherapeutic agents are relatively ineffective (3, 4). An important goal would be to understand the characteristics of transformed hepatocytes better and pursue strategies for early in vitro and in vivo diagnosis.

In this regard, we have prepared several libraries of monoclonal antibodies against a newly developed (5) and other established HCC cell lines. Such antibodies have been tested for their specificity towards HCC, and several have been shown to recognize antigenic determinants that appear thus far almost exclusively on transformed hepatocytes. These antibodies, anti-HCC, have been employed in the construction of highly sensitive and specific radioimmunoassays that may detect "hepatoma-associated" antigens. In addition, one anti-HCC monoclonal antibody appears suitable for in vivo localization of tumor by nuclear imaging in a nude mouse system. The observed specificity of these antibodies, in conjunction with the characterization of their antigens, allow us to conclude that these antibodies will be useful in further clarifying the properties of transformed hepatocytes.

Methods

Hepatoma cell lines. PLC/PRF/5, SK Hep-1, and FOCUS HCC lines used for immunization of mice were maintained in Earle's modified Eagle's medium (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum inactivated at 56°C, 10 μM nonessential amino acids, 1,000 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were harvested from the monolayer cultures by washing three times with phosphate-buffered saline (PBS) followed by treatment with versene buffer in the absence of trypsin. Single cell suspensions thus obtained were used for immunization of BALB/c mice. All HCC cells were tested for mycoplasma by DNA staining and culture and found to be negative (kindly performed by the Mycoplasma Testing Laboratory, Massachusetts General Hospital).

Immunization and fusion protocols. Primary immunizations were accomplished with 4.0×10^6 cells/ml in 50% complete Freund's adjuvant and were injected intraperitoneally into female BALB/c mice. The secondary immunizations were performed 3 d before cell fusion by an intravenous inoculation of 4.0×10^6 cells/200 μl in PBS. The interval between primary and secondary immunizations varied between 6 and 10 wk. Splenocytes were fused with the NS1 myeloma cell line. The maintenance and selection of hybridomas were as previously described (6).

Selection of hybridomas with anti-HCC activity. Two radioimmunoassays (RIAs) were performed to identify hybridomas that produce

antibodies with anti-HCC binding activity. In the first assay, 100,000 of the respective HCC target cells in 100 μl of Earle's modified Eagle's medium were incubated with 100 μl of cell culture supernatant from 70% confluent hybridoma lines for 1 h at 37°C. Cells were then washed three times with Hanks' balanced salt solution and resuspended between washings by gentle agitation followed by centrifugation at 1,200 rpm for 5 min. Resuspended cell pellets were subsequently incubated with 100,000 cpm ^{125}I sheep antimouse F(ab')$_2$ (New England Nuclear, Boston, MA) in 100 μl of PBS with 1% bovine serum albumin for 0.5–1.0 h at 37°C. In the second assay cells were grown on glass coverslips, washed three times with PBS, fixed via immersion into −80°C ethanol/acetone mixture (9:1), and incubated with 1:100 dilutions in PBS of ascites fluid preparations containing the anti-HCC antibodies of interest as well as nonrelevant monoclonal antibodies of the same isotype for 1.0 h at room temperature. The slides were washed three times with PBS and incubated for 1.0 h at room temperature with a 1:20 dilution of fluorescein-conjugated goat anti-mouse IgG F(ab)$_2$ (Cappel Laboratories, Cochranville, PA). After washing three times with PBS, the coverslips were mounted onto glass slides with a 9:1 glycerol/PBS solution and observed under a Zeiss microscope with an epifluorescent attachment (Carl Zeiss, Inc., Thornwood, NY). Other normal human tissues including liver were prepared as snap-frozen specimens in liquid nitrogen, cryostat-sectioned, and processed in an identical fashion.

Purification and iodination of anti-HCC antibodies. Monoclonal antibodies from double-cloned cell lines of the IgG subclass were purified for further study by using a Sepharose 4B staphylococcal protein A-affinity column (Pharmacia Fine Chemicals, Piscataway, NJ). 1 ml of centrifuged, filtered ascites fluid was placed on the column at pH 8.0. The ascites fluid was allowed to remain on the column without washing for 30 min at 4°C, whereafter the column was washed with PBS at pH 8.0, and mouse IgG isotypes were eluted with various buffers according to a previously established technique (7). Purified antibodies thus obtained were dialyzed overnight against twice-normal concentrated saline and the protein concentration was determined by the method of Lowry et al. (8). Iodination of the antibodies to a specific activity varying between 8 and 10 μCi/μg with ^{125}I was accomplished by the Iodogen method.

Cell surface membrane binding studies. Direct binding of ^{125}I-labeled anti-HCC antibodies was performed by incubating HCC and control cell lines in suspensions as described above. In brief, 100,000 cpm ^{125}I anti-HCC in 100 μl of PBS-1% BSA was incubated with cells at 37°C for 1 h. Cells were washed and counted. Finally, we also employed ^{125}I-labeled nonrelevant IgG$_{2a}$ monoclonal antibody designated TH-1011 as a control.

Development of RIAs. Attempts were made to establish RIAs for in vitro detection of antigens related to HCC cells by using previously characterized anti-HCC monoclonal antibodies. These studies were performed as follows: ¼-in polystyrene beads (Precision Plastic Ball Co., Chicago, IL) were coated by a 1:500 dilution in PBS of ascites fluid containing the antibody of interest. After 16 h incubation of the solid-phase support with ascites fluid at 20°C, the beads were washed three times with distilled water and then incubated with culture supernatants derived from confluent HCC cells and other control cell lines. The radiolabeled indicator antibody consisted of a ^{125}I anti-HCC monoclonal identical to the antibody linked to the solid-phase support. Radiolabeled indicator antibody diluted in PBS-50% fetal calf serum (100 μl) and containing 100,000 cpm (sp act 8–10 μCi/μg) with nonspecific mouse monoclonal IgG (20 μg/100 μl) was added to the assay. The RIAs were performed either in the "forward or simultaneous sandwich" mode (9) with incubation times varying from 0.5 to 24.0 h. Incubation temperatures ranged from 4 to 45°C. After incubation with the test samples, the beads were washed three times with distilled water and counted in a gamma well counter.

Competitive inhibition studies. We determined whether the various anti-HCC antibodies recognized the same, closely related, or distinct epitopes on the HCC cell surface by competitive binding experiments. For this procedure HCC cells were fixed to a solid-phase support as

described (10). ^{125}I-labeled monoclonal anti-HCC antibodies (100,000 cpm) were incubated with increasing amounts of unlabeled homologous antibody to generate an inhibition curve. Next, the unlabeled homologous antibody was substituted for by a different unlabeled anti-HCC antibody and the counts per minute bound to the cells was determined at increasing antibody concentrations. If, for example, no inhibition of binding was observed under these conditions, we may presume that the second unlabeled antibody recognizes a separate and distinct determinant on HCC cells.

Identification of cell surface antigens. Human hepatoma-associated cell surface antigens were partially characterized by using the method described by Soule et al. (11). The HCC cells of a confluent 75-cm^2 monolayer culture were radiolabeled with ^{125}I by the lactoperoxidase method (12). Subsequently, the cells were washed three times with ice-cold PBS and then solubilized in the flasks at 0°C for 0.5 h by the addition of 3.0 ml of 1% Nonidet P-40, 20 mM Tris-HCl, pH 8.3, 140 mM NaCl, and 100 U trasylol/ml. The cell lysate was spun at 40,000 rpm for 30 min in a 50 Ti rotor to remove debris and then 0.5-ml aliquots of the supernatant were immunoprecipitated with individual anti-HCC antibodies by addition of 50 μl of ascites fluid. Immune complexes were captured on fixed-killed Staph A cells (Boehringer-Mannheim Diagnostics, Houston, TX). These cell pellets were resuspended in 100 μl of SDS polyacrylamide gel electrophoresis (PAGE) buffer, heated at 100°C for 2.5 min, and then clarified at 1500 g for 5.0 min. The supernatant fractions were electrophoresed on a 10% polyacrylamide slab gel (13). Gels were fixed, stained, destained, and/or dried and autoradiographed for 12.0 h to 2.0 days by using Kodak x-ray film (Eastman Kodak Co., Rochester, NY).

Additional characterization of HCC-associated antigens was attempted when noniodinated cells were solubilized and centrifuged as described above. The clarified lysates were placed on affinity columns constructed with monoclonal anti-HCC antibodies bound to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) at pH 7.4 for 1 h. The columns were then extensively washed with PBS, pH 2.6, and 4.0 M urea. 2-ml fractions were collected and assayed for binding activity by using the RIAs described above. Peak binding fractions were dialyzed against PBS and concentrated with a Micro devices Pro-Di-Con (Jenkintown, PA) (14). Concentrated samples (25 μl) were electrophoresed under reducing conditions on 10% polyacrylamide gels.

Nuclear imaging studies. Intact native anti-HCC antibody or prepared Fab (15) fragments were radiolabeled with 1.5 mCi [131]I (Amersham Corp., Searle Div., Arlington Heights, IL) using the Iodogen technique. Female BALB/c nude mice were injected in the left shoulder with either 1 × 10^8 PLC/PRF cells in 0.2 ml of PBS, or with a 2-mm^3 piece of an explanted PLC/PRF/5 tumor obtained from an existing tumor-bearing mouse. Tumors grew to a size of 1–3 cm (diam) after ~4 wk and were subsequently subjected to nuclear imaging studies.

Tumor-bearing mice were injected via the tail vein with a 50–100 μCi ^{131}I intact monoclonal antibody or the corresponding Fab. Imaging was performed at 6, 12, 18, 24, 48, and 72 h with an Anger camera (1.25-cm crystal) equipped with a 5-mm aperture pinhole collumator. All data were recorded on a dedicated computer system and stored on floppy disks.

Biodistribution of the radiolabeled antibody at the conclusion of imaging was determined in blood and tumor and comparisons were made to gastrointestinal tract, stomach, liver, spleen, kidneys, heart, and lungs. Before measurement of tissue uptake of ^{131}I-labeled antibody, all organs were washed extensively with PBS. Biodistribution of isotope was calculated and expressed as counts per minute per gram of tissue.

Results

Production and characterization of anti-HCC antibodies. We used single-cell suspensions, removed from cell cultures without proteolytic enzyme treatment, in an attempt to develop anti-

HCC monoclonal antibodies to native cell surface membrane-associated antigens. An immunization protocol was employed where the primary immunization was intraperitoneal followed by a 4–10-wk maturation period. Next an intravenous boost of intact whole cells was given. This approach allowed us to establish several libraries of anti-HCC monoclonal antibodies. 35 anti-HCC antibody-producing hybridoma cell lines were developed by using PLC/PRF/5 as the immunizing cell type.

Experiments were performed to compare the properties of monoclonal antibodies produced against PLC/PRF/5 cells to others developed against different HCC cell lines. Thus a library of anti-HCC antibodies to a HCC cell line designated FOCUS developed in our laboratory (5) and SK-Hep-1 cells were prepared. 62 hybridoma cell lines producing anti-HCC antibodies to both FOCUS and SK-Hep-1 HCC cell lines were identified by high binding activity in the RIA (data not shown). Indeed, a primary immunization with intact cells followed by a maturation period of 6–10 wk before the second intravenous boost with a whole-cell suspension was found to be a successful schedule for production of anti-HCC antibodies.

The specificity of the antibodies was assessed by binding studies to a panel of cells as shown in Table I. These experiments allowed us to select antibodies of better-defined specificity for further characterization as described below. For example, the anti-PLC/PRF/5 antibody designated P215457 demonstrated high binding activity not only to four human hepatoma cell lines namely, SK-Hep-1, PLC/PRF/5, FOCUS, and Mahlavu but also to two other rat hepatoma cell lines (HTC-41 and MH1C1). In contrast, this antibody did not bind to the human embryo lung fibroblast (HELF). On the other hand, another anti-PLC/PRF/5 antibody designated PM4E9917 recognized antigens on all four human HCC cell lines, but not two of three rat hepatomas or other human cell lines except SW480, a human colorectal adenocarcinoma. Other antibody-binding patterns were of interest. Antibody 10-61-25 produced against FOCUS demonstrated high binding to FOCUS, SK-Hep-1, and Mahlavu, but no binding activity was observed to PLC/PRF/5, rat hepatomas, or the other two human cell lines. Yet another antibody, such as II45 developed by immunization with SK-Hep-1 cells, recognized antigens on SK-HEP-1 and Mahlavu but lacked binding activity to a panel of other human cells as shown in Table I. Thus monoclonal antibodies produced against the various HCC cell lines may show differential binding activity that allows one to choose antibodies of interest with respect to recognition of antigenic determinants that appear common to transformed hepatocytes.

Table I. Representative Examples of Specificity of Anti-HCC Antibodies to Various Cell Lines as Shown by Direct Binding Studies

| Antibody | Culture supernatant dilutions | PLC/PRF/5 | Human hepatoma | | | Rat hepatoma | | Controls | |
			SK-HEP-1	Focus	Mahlavu	MH_1C_1	HTC41	SW480‡	HELF
PM4E9917*	10^{-1}	3.0	8.1§	3.0	3.5	1.2	1.5	1.1	1.1
	10^{-2}	2.0	4.66	1.9	2.4	1.1	1.1	1.0	1.0
P232524	10^{-1}	8.3	12.0	13.0	4.2	1.1	1.1	3.1	1.0
	10^{-2}	9.0	15.0	14.0	4.8	1.1	1.0	4.6	1.0
P235F3	10^{-1}	11.0	23.0	14.0	10.0	1.4	2.6	3.1	0.9
	10^{-2}	13.0	21.0	14.0	10.0	1.3	2.5	4.6	1.0
P29759	10^{-1}	10.0	16.0	11.0	9.1	1.9	3.7	3.8	0.9
	10^{-2}	12.0	17.0	10.0	10.0	1.2	1.8	6.0	1.0
P215457	10^{-1}	12.0	24.0	15.0	12.0	3.9	7.9	4.2	1.3
	10^{-2}	16.0	28.0	20.0	14.0	3.2	8.0	8.0	1.0
10-61-25	10^{-1}	1.0	26.0	14.0	19.0	0.8	0.9	1.3	1.1
	10^{-2}	1.0	23.0	13.0	19.0	1.0	1.0	2.1	0.9
II45	10^{-1}	1.1	15.0	0.8	13.0	0.8	0.8	0.4	0.8
	10^{-2}	0.9	5.0	0.9	4.9	1.0	1.0	0.6	0.9
II101	10^{-1}	2.0	15.0	2.5	13.0	0.8	0.9	0.5	1.2
	10^{-2}	1.2	6.2	1.3	5.4	1.0	0.9	0.9	1.0
II110	10^{-1}	5.6	12.0	9.0	1.0	1.1	0.9	—	1.0
	10^{-2}	2.6	6.0	5.4	1.0	1.1	1.0	—	1.0
II158	10^{-1}	3.3	13.0	10.0	11.0	1.1	0.9	—	0.4
	10^{-2}	2.2	16.0	14.0	13.0	1.0	1.3	—	1.0

* PM4E9917, P232524, P235F3, P29759, and P215457 are anti-PLC antibodies; 10-61-25 is an anti-FOCUS antibody; II45, II101, II110, and II158 are anti-SK-Hep-1 antibodies.   ‡ SW480 is a human adenocarcinoma of the colon and HELF is a human embryo lung fibroblast cell line. § S/N (signal/noise) defined as the counts per minute bound by culture supernatant containing the anti-HCC antibody divided by control culture supernatant (see Methods). A positive result is defined as a S/N of >2.0.

It was desirable to select anti-HCC antibodies for possible in vivo and in vitro immunodiagnosis. Antibodies produced against PLC/PRF/5 and designated PM4E9917 (IgG$_{2a}$), P232524 (IgG$_1$), P235F3 (IgG$_{2b}$), P29759 (IgG$_1$), and P215457 (IgG$_{2a}$) were selected from the library of monoclonal anti-HCC antibodies for further studies in that such antibodies recognize common antigenic determinants present in all four human hepatoma cell lines (Table I). Further, these anti-HCC antibodies demonstrated high binding activity with serial dilutions of culture supernatants and ascitic fluids to such cells, thus leading us to believe that they may possess high affinities for their antigenic determinants (data not shown); all were found to be of the IgG class.

The binding characteristics of these five anti-HCC antibodies were further explored after isolation and purification from ascitic fluid by staphylococcal A affinity chromatography. Monoclonal antibodies were labeled with ^{125}I to a specific activity varying between 8 and 10 μCi/μg. Maximal binding of antibody to the cells was evident within the 1st h of incubation followed by a gradual decrease in binding activity over an 18-h period. Similar kinetics of binding were obtained with the other five anti-PLC/PRF/5 antibodies (data not shown). The rapid near saturation binding of P215457 during the 1st h of incubation suggests that the affinity of the antibodies for their antigenic determinant may be high. Furthermore, 30–45% of the input (100,000 cpm) were bound to the PLC/PRF/5 cells suggesting a high representation of this antigenic determinant on the cell surface. We took advantage of the binding properties in in vivo nuclear imaging studies as shown below.

After having established that the binding kinetics of the anti-HCC antibodies were rapid, we then compared the maximal binding activity of five radiolabeled antibodies to each other by incubating the antibodies with PLC/PRF/5 cells in suspension. In this experiment 100,000 cpm of each anti-PLC/PRF/5 antibody and a nonrelevant control antibody was incubated with PLC/PRF/5 cell in suspension for 1 h at 37°C. Antibody P215457 demonstrated the highest binding activity with 34% of the added radioactivity bound after 1 h. The other antibodies, namely P29759, P232524, PM4E9917, and P235F3, showed lesser but highly significant degrees of binding activity to PLC/PRF/5 cells in suspension (27, 16, 11, and 3%, respectively).

We further evaluated the specificity and characteristics of the antibodies for HCC membrane antigens by using an indirect immunofluorescent technique. Monoclonal antibodies P215457 and PM4E9917 demonstrated strong cell surface membrane fluorescence with PLC/PRF/5 cells as shown in Fig. 1, whereas P232524 stained primarily a cytoplasmic antigenic determinant (data not shown). Table II depicts the immunofluorescent results of these three antibodies tested on 38 other human and nonhuman cell lines in order to assess the specificity of the membrane staining. It was striking that all three antibodies recognized cell surface antigens on the four human hepatomas as well as some of the rat hepatoma cell lines. The only nonhepatoma cell line that demonstrated
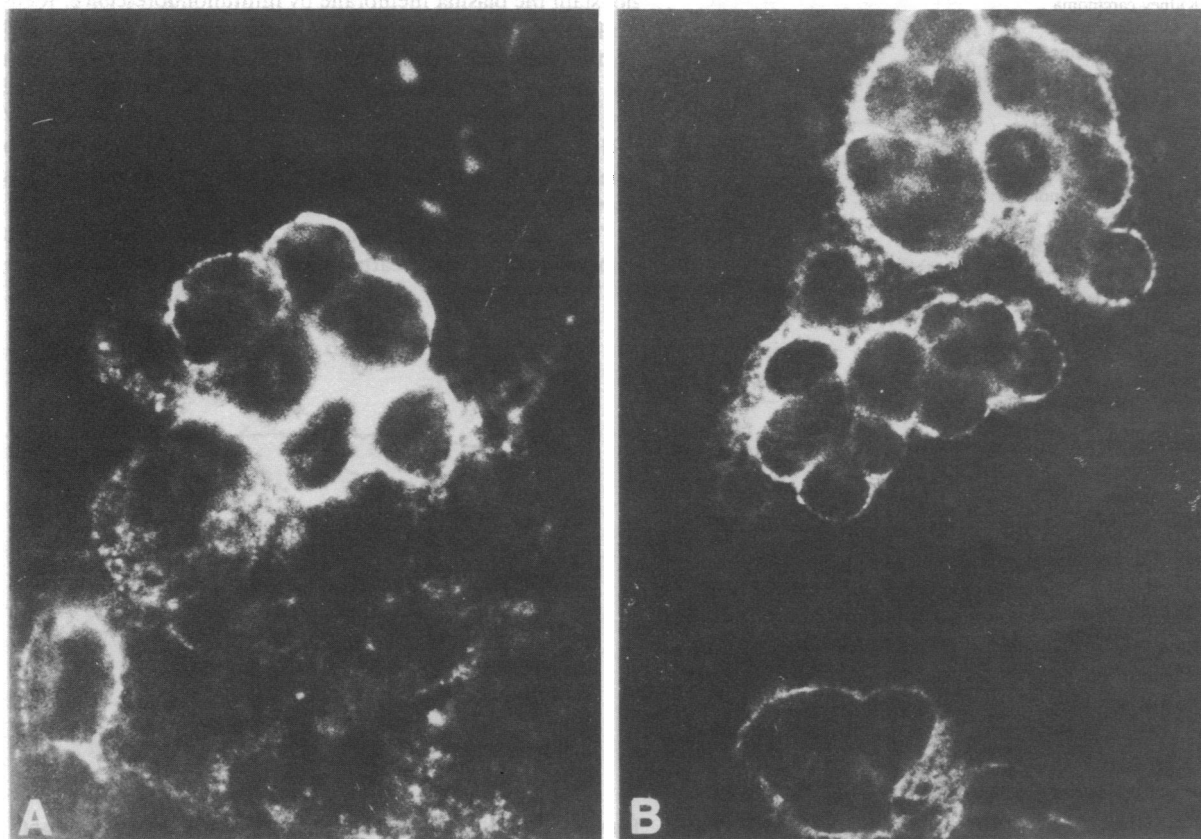

Figure 1. Indirect immunofluorescent staining of cell surface membrane associated antigen(s) in PLC/PRF/5 (*A*) and Mahlavu (*B*) HCC cells by monoclonal antibody P215457.

Table II. Results of Specificity Testing by Immunofluorescence of Other Tumor Cell Lines

Cell line*	Tissue of origin	Monoclonal antibodies‡		
		P215457	P232524	PM44E9917
PLC/PRF/5		+	+	+
MAHLAVU	Hepatomas	+	+	+
SK-Hep-1		+	+	+
FOCUS		+	+	+
MH1C1		+	−	−
HTC-41	Rat hepatomas	+	+	+
McA-RH7777		−	−	−
SW480	Colon adenocarcinomas	+	+	+
CACO-3		−	−	−
LS180		−	−	−
WiDr		−	−	−
COLO320		−	−	−
SW1116		−	−	−
SK-CO-1		−	−	−
CAPAN-1	Pancreatic adenocarcinomas	−	−	−
CAPAN-2		−	−	−
BT-20	Breast adenocarcinoma	−	−	−
A704	Kidney adenocarcinoma	−	−	−
CALU-3	Lung adenocarcinoma	−	−	−
HUTU-80	Duodenal adenocarcinoma	−	−	−
AN3CA	Endometrial adenocarcinoma	−	−	−
CAOV-3	Ovarian adenocarcinoma	−	−	−
A498	Kidney carcinoma	−	−	−
5637	Bladder carcinoma	−	−	−
Hela	Epithelioid carcinoma of cervix	−	−	−
Hep-2	Epidermoid carcinoma of larynx	−	−	−
A427	Lung carcinoma	−	−	−
DU-145	Prostate carcinoma	−	−	−
SK-UT-1	Mesodermal carcinoma	−	−	−
JEG-3	Choriocarcinoma	−	−	−
U87-MG	Glioblastoma-astrocytoma	−	−	−
C-33A	Cervical carcinoma	−	−	−
SK-Mel-5	Melanoma	−	−	−
CHANG	Liver derived	−	−	−
DET 551	Embryonic skin	−	−	−
HELF	Embryonic lung fibrosis	−	−	−
HL	Human lymphocytes	−	−	−
3T3	Mouse fibroblasts	−	−	−

* All cell lines are of human origin unless otherwise stated.
‡ +, positive results; −, negative results.

staining was a colorectal adenocarcinoma (SW480). Thus, all other malignant cell lines, and in addition, cut frozen tissue from two pancreatic adenocarcinomas with liver metastasis as well as cut frozen sections of normal human liver, pancreas, heart, kidney, skeletal muscle, gastrointestinal tract, leukocytes and erythrocytes, platelets, lung, and brain, were negative for immunofluorescent staining (data not shown). These results suggest that the three monoclonal antibodies described here have specificity for human hepatoma-associated antigens.

It was of interest to determine if the anti-PLC/PRF/5 antibodies recognized the same, closely related, or separate

antigenic determinants on the cell surface membrane of PLC/PRF/5 cells as measured by competitive binding studies shown in Fig. 2. In these experiments, addition of increasing amounts of unlabeled PM4E9917 to ^{125}I-PM4E997 in the presence of PLC/PRF/5 cells demonstrated inhibition of binding of ^{125}I PM4E9917 to PLC/PRF/5 cells. If, however, PM4E9917 was replaced in the experiment by unlabeled P215457, P232524, or TH-1011 (control antibody), no inhibition of binding of radiolabeled antibody was observed. Similar results were obtained when P232524 and P215457 served as the radiolabeled antibodies in the competitive inhibition experiments (data not shown). From these results, we are led to believe that the three antibodies recognize separate and distinct surface membrane determinants on PLC/PRF/5 cells.

Partial characterization of hepatoma-associated antigens. Tests were performed with monoclonal anti-PLC antibodies for binding activity to α-fetoprotein, hepatitis B surface antigen (HBsAg), and carcinoembryonic antigen by using commercially available kits (Abbott Laboratories, No. Chicago, IL); there was no binding activity of the antibodies to these proteins.

In an attempt to define further the characteristics of the cell surface antigen(s), cell membrane proteins were radiolabeled by the lactoperoxidase method as previously described (15). Thus ^{125}I PLC/PRF/5, Mahlavu, HTC-41 (rat hepatoma) cells, and HELFs were studied. As shown by the autoradiographs in Fig. 3, PM4E9917 precipitates a protein with a molecular weight of ~65,000 (lane 1), whereas P215457 recognizes a cell surface protein of ~50,000 mol wt (lane 3). Antibody P232524 did not precipitate a ^{125}I-labeled cell surface membrane protein (lane 2). It is noteworthy that this antibody also did not stain the plasma membrane by immunofluorescence. Identical antigens with respect to molecular weight were identified on the Mahlavu cell line by PM4E9917 and P215457 (data not shown). Of considerable interest was the finding that the two proteins recognized by PM4E9917 and P215457 on PLC/PRF/5 and Mahlavu human hepatoma cell lines were also detectable on the rat hepatoma cell surface membranes (lanes 6 and 9, respectively).

The specificity of the antigen–antibody interaction was demonstrated by the treatment of ^{125}I-labeled cell membrane
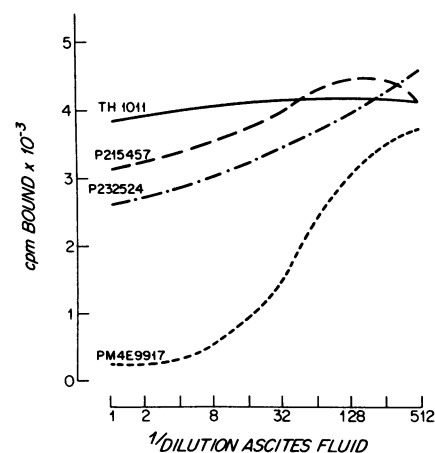

Figure 2. Competitive inhibition experiment demonstrating that antibodies P215457, P232524, and PM4E9917 recognize distinct and separate antigenic determinants on PLC/PRF/5 HCC cells. TH-1011 represents a nonrelevant control monoclonal antibody.
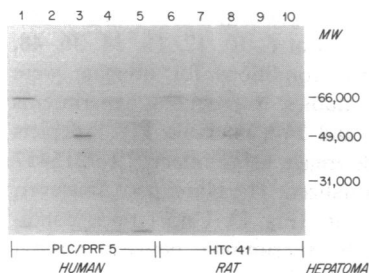
Figure 3. Autoradiograph of 10% SDS-PAGE of hepatoma-associated antigens. Lane *1*, immunoprecipitate of PLC/PRF/5 extract with anti-HCC PM4E9917 demonstrating a protein of ~65,000 mol wt; lane *2*, immunoprecipitate of PLC extract with anti-HCC P232524; lane *3*, immunoprecipitate of PLC/PRF/5 cells by anti-HCC P215457 demonstrates a protein antigen of 50,000 mol wt; lane *4*, immunoprecipitation of PLC/PRF/5 extract by anti-HCC P29759 demonstrating a faint band at 64,000 mol wt; lane *5*, immunoprecipitate with nonrelevant monoclonal antibody TH-1011 demonstrating the specificity of the antigen–antibody interaction technique; lanes *6–10*, the same antibodies as represented in lanes *1–5* where a rat hepatoma extract has been studied in an identical manner to PLC/PRF/5 cells. Note that the same protein antigens observed with PLC/PRF/5 cells were also detected with anti-HCC antibodies PM4E9917, P215457, and P29759. Identical results were also observed with the Mahlavu HCC cell line (data not shown). MW, molecular weight.

proteins from PLC/PRF/5 and HTC-41 with a nonrelevant monoclonal antibody (TH1011) as shown in lanes 5 and 10. Finally, as an additional control ^{125}I-labeled HELF cells were radiolabeled and immunoprecipitated by the two anti-PLC/PRF/5 monoclonal antibodies; no precipitation of radiolabeled proteins was observed by autoradiography (data not shown).

Additional experiments were performed by incubating PLC/PRF/5, Mahlavu, FOCUS, and SK-Hep-1 HCC and control cells in glucosamine-free medium with [^{3}H]glucosamine. We were unable, under these conditions, to demonstrate incorporation of [^{3}H]glucosamine into any of the immunoprecipitates.

In vitro assays. After having identified and partially characterized hepatoma-associated cell surface proteins by anti-HCC monoclonal antibodies, studies were performed in an attempt to detect soluble antigens in cell culture supernatants derived from both human and rat hepatoma, as well as other nonhepatic human tumor cell lines. We developed, therefore, a series of RIAs that employed various combinations of antibodies PM4E9917, P232524, P241AE, P235F3, P29759, and P215457 both in a "simultaneous" and forward sandwich mode (9). In general, the simultaneous sandwich assay design was found to be the optimal assay configuration with respect to sensitivity in a homologous system where the antibody bound to the solid-phase support and the radiolabeled indicator antibody are the same. High binding activity detected in culture supernatants derived from human hepatoma cell lines was only observed with PM4E9917, P232524, and P215457 monoclonal antibodies that used the simultaneous sandwich RIAs; we pursued the characteristics of the binding activity in greater detail.

Fig. 4 depicts a representative experiment which employs a homologous simultaneous sandwich RIA that uses monoclonal antibody P215457. High binding activity was exhibited present in cell culture supernatants derived from SK Hep-1 and Mahlavu HCC cell lines. In addition, a high binding activity was demonstrated in the cell culture supernatant of the rat hepatoma line HTC-41. A low level of binding activity was also observed with another rat hepatoma cell line MH1C1. No binding activity was demonstrated in the cell culture
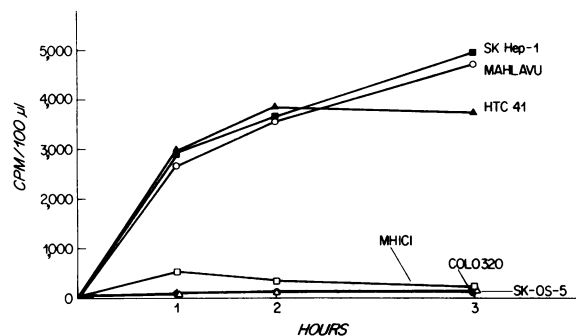

Figure 4. "Simultaneous sandwich" monoclonal RIA with anti-HCC antibody P215457. 100 μl of cell culture supernatant from two human hepatoma cell lines (SK-Hep-1 and Mahlavu) and two rat hepatoma cell lines (HTC-41 and MHICI) were incubated for 1–3 h. Note the high binding activity exhibited by two human and one rat hepatoma lines. Low binding activity was observed with the other rat hepatoma line. Controls were cell culture supernatants from two other human carcinoma cell lines.

supernatants derived from the colorectal cell line COLO-320 or the glioblastoma line SK-0S-5.

Similar observations were obtained with antibody P232524 by using an identical simultaneous sandwich homologous assay system, as shown in Fig. 5. High binding activity was observed in cell culture supernatant from two HCC cell lines but not from control cells. Attempts were made to optimize the binding activity observed in the RIAs. Previous studies (Figs. 4 and 5) had established that near-maximal binding of antibody to antigen in the RIA was apparent after a 4-h incubation. Fig. 6 demonstrates that when using P215457 maximal binding activity occurs at 4°C. It is also apparent that the magnitude of binding activity is a direct function of temperature of incubation. It is somewhat surprising, however, that higher temperatures were associated with lower binding activity in the RIA. Fig. 6 also illustrates the specificity of P215457 for a human and/or rat hepatoma associated antigen(s) in cell culture supernatants.

It was of interest to isolate and characterize the binding activity in HCC cell culture supernatants, and we took advantage of the properties of antibodies P215457 and P232524. Thus, these two antibodies were coupled to cyanogen bromide-activated Sepharose 4B for isolation of antigenic activity by affinity chromatography. The affinity-purified and concentrated protein had been shown to have high binding activity in the RIAs before subjecting this material to SDS-PAGE. Fig. 7 demonstrates the presence of a 45,000-mol wt protein (lane 4) in cell culture supernatant derived from PLC/PRF/5. It is of interest that a similar antigen was likewise identified by P215457
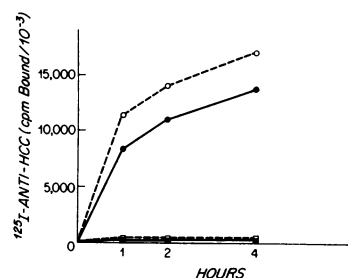

Figure 5. "Simultaneous sandwich" monoclonal RIA with anti-HCC PM4E9917 demonstrating high binding activity in cell culture supernatant from PLC/PRF/5 (●) and Mahlavu (○) and not from two carcinomas of the colon and brain.
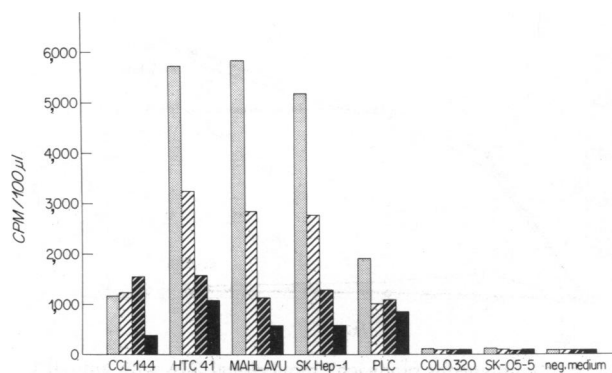
Figure 6. Effect of temperature on binding activity observed in cell culture supernatant by a simultaneous sandwich RIA using anti-HCC monoclonal antibody P215457. Positive binding activity was detected in all human and rat hepatoma cell lines, namely, PLC/PRF/5, Mahlavu, SK-Hep-1 and HTC-41, respectively. There was an enhancement in binding activity with decreasing temperature. ■, 45°C; ▨, 37°C; ▤, 20°C; ▥, 4°C.

on the cell surface of both human and rat hepatoma cell lines (see Fig. 2). We were, however, unable to demonstrate an antigen by this technique with antibody P232524 (Fig. 7, lane 3).

In vivo immunoassays. Monoclonal antibody P215457 was selected for in vivo imaging studies of HCC because of the following special properties: (a) the antibody demonstrated the highest binding activity to four human hepatoma cell lines (Table I); (b) the kinetics of binding was rapid—maximal binding occurred after a 1-h incubation; (c) immunofluorescent studies demonstrated intense cell surface membrane immunofluorescence (Fig. 1); and (d) there was a sufficient concentration of P215457 in ascitic fluid (5–7 mg/ml) to prepare Fab fragment. Imaging of HCC may present several special problems because of its primary hepatic origin. Numerous attempts were made in the nude mouse system to image with ^{99m}Tc and ^{111}In coupled to P215457 by diethylene triamine penta-acetic acid (DTPA). However, liver and spleen uptake of the conjugate was as high as that found in the tumor (data not shown). We therefore abandoned this approach and proceeded to perform imaging studies with ^{131}I-labeled P215457.

Two types of imaging experiments were performed. The PLC/PRF/5 tumors were continuously maintained by the explant technique in the right or left shoulder of nude mice. When such tumors reached a size of 0.5 cm or larger (diam), imaging studies were performed with intact ^{131}I-P215457 or the prepared Fab fragment. Each mouse received 50–100 μCi

of ^{131}I-antibody via a tail vein injection (sp act 14–20 μCi/μg). Imaging studies were performed at 6, 10, 12, 18, 24, 36, 48, and 72 h. Once the optimal conditions for imaging were established by preliminary studies, subsequent experiments were performed only at 10- and 24-h intervals. Fig. 8 depicts a representative radionuclide image using intact ^{131}I-P215457 at 24 h. The tumor is well visualized. The blood pool, however, contains significant radioactivity (Fig. 8). Under these conditions, it would be difficult to discriminate between normal liver, tumor, and the blood pool. Similar results were observed at later time periods, e.g., 36, 48, and 72 h (data not shown). Biodistribution studies performed with the intact antibody at 24 h are shown in Table III. The highest radioactivity was present in the blood followed by tumor, spleen, and heart and in general confirm the radionuclide imaging results.

In the second set of studies, the experimental conditions were changed and attempts were made to image with the Fab fragment of P215457. This prepared fragment had a molecular weight of 45,000 on SDS-PAGE and appeared as a single protein band with little, if any, contamination with intact antibody or F(ab)₂ fragment. In addition, the immunoreactivity of the Fab fragment was reevaluated by direct binding studies to PLC/PRF/5 cells in suspension before injection into the nude mouse that bore the tumor. The preparation of the Fab fragment resulted in an ∼10-fold reduction in immunoreactivity when compared to the native antibody (data not shown). Despite this significant reduction in immunoreactivity in vitro, Fig. 9 shows a representative serial imaging study with ^{131}I-Fab. In this experiment 50 μCi of ^{131}I-P215457 Fab was injected into the tail vein. Fig. 9 *B* shows the nude mouse before imaging and demonstrates the size of the tumor. Fig. 9 *A* is an image of tumor localization at 10 h showing substantial uptake in the human hepatoma but a relatively high background blood pool. Fig. 9 *C* is the same mouse imaged at 24 h. There is a substantial improvement in tumor localization due to the clearance of ^{131}I-Fab from the blood pool. Fig. 9 *D* is an image at 24 h of another animal bearing a nonhepatoma tumor in the left shoulder again demonstrating the specificity of ^{131}I-P215457 Fab for human hepatoma localization. Table III depicts the biodistribution of ^{131}I-Fab in the various tissues. It was of interest that the uptake of ^{125}I-Fab in the tumor compared to normal liver and blood pool was 9:1 and 4:1, respectively. We speculate that the high uptake in the stomach may be due in part to dehalogenation of the Fab and concentrations of free ^{131}I in place of CL⁻ in the lumen of the stomach during acid secretion.

Discussion

In the present investigation we have established several libraries of monoclonal antibodies directed towards antigenic determinants present on HCC cell lines to understand the properties of transformed hepatocytes better. We believe that the preparation of cells before immunization, the route of immunization, and the interval between primary and secondary immunizations were important features in establishing such antibodies with the desired properties for the development of experimental in vitro and in vivo immunoassays. Indeed, cells were removed from tissue culture dishes without proteolytic enzyme treatment in an attempt to preserve native cell surface antigenic determinants. Next, an interval of 6–10 wk was allowed between
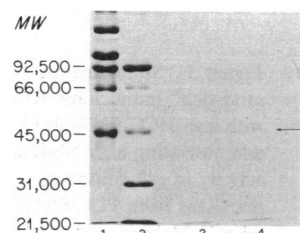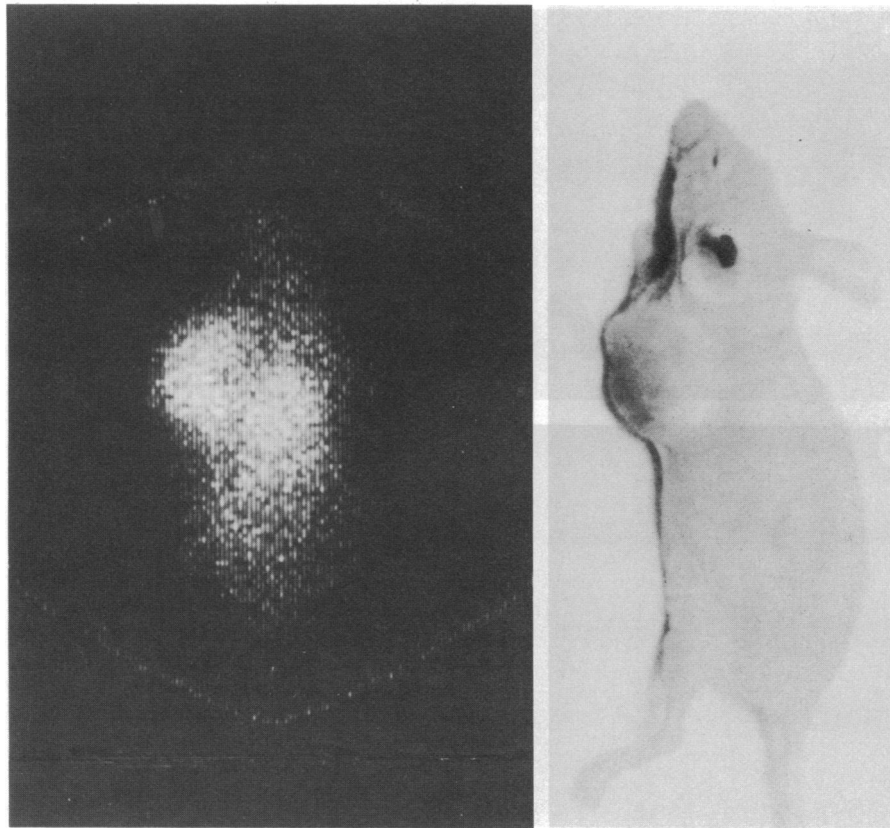

Figure 7. 10% SDS-PAGE of affinity purified HCC-associated antigen by monoclonal antibodies P232524 and P215457. Note that a 45,000-mol wt protein was isolated from PLC/PRF/5 cell culture supernatant (lane *4*) by using antibody P215457 on the affinity column. Lane 3 represents affinity-purified material that uses antibody P232524. Lanes *1* and *2* are molecular weight standards. MW, molecular weight.

Figure 8. Radionuclide imaging of HCC PLC/PRF/5 tumor in nude mice with intact ^{131}I-P215457. In this experiment ~50 μCi of monoclonal antibody was injected (200 μl) into the tail vein. Images were performed 24 h later. Note uptake of antibody into the tumor tissue. However, the blood pool radioactivity is very high (also see Table III).

primary and secondary immunization in an attempt to generate high affinity antibodies (6). Most importantly, the secondary boost was accomplished intravenously with a nonproteolytic enzyme-treated single cell suspension. One disadvantage of the immunization protocol is the loss of immunized mice after the i.v. boost presumably due in part to the impediment of blood flow in the microcirculation by clumps of large human hepatoma cells.

It is noteworthy that others have produced monoclonal antibodies to HCC lines (6). In one study, monoclonal antibodies to a HCC line designated Hep G-2 were produced after two intraperitoneal injections with trypsinized cells spaced 10 d apart. Two antibodies named 833-1C4 clone G1 and

Table III. Biodistribution of ^{131}I-P215457 in Various Organs 24 h After Tail Vein Injection into Human Hepatoma-bearing Nude Mice

Organ	Whole antibody	Fab
	cpm/g tissue (×10⁻⁵)	*cpm/g tissue (×10⁻⁵)*
Blood	10.85	0.77
Gut	1.01	0.14
Stomach	1.04	3.09
Liver	1.64	0.36
Spleen	2.88	0.64
Kidney	1.87	1.13
Lung	1.95	0.13
Heart	2.06	0.51
Tumor	3.72	3.15

833-2B2 clone F1 were selected for characterization and further study. Clone G1 identified a 115,000-mol wt glycoprotein on Hep G-2 cells. This antibody, however, also reacted with colorectal carcinoma-derived cell lines and, in addition, recognized an antigenic determinant present on some fibroblasts, mammary and ovarian carcinomas, melanoma, and epidermoid carcinoma cell lines. Clone F1 identified an antigenic determinant on several Hep G-2-derived proteins of 230,000, 79,000, 23,000, and 20,000 mol wt and also cross-reacted with an epitope present on several colorectal, mammary, and ovarian carcinoma cell lines. In addition, this antibody recognized a determinant on normal human kidney tubular epithelium (16). Another preliminary report describes the production of three monoclonal antibodies designated K_1, K_2, and K_3 to PLC/PRF/5 cells. Antibody K_1 cross-reacted with a number of other tumor cell lines, whereas K_2 and K_3 apparently reacted with only PLC/PRF/5 cells and identified a 200,000- and 47,000-mol wt protein, respectively. Details regarding further characterization of these antibodies are not yet available (17).

Of particular interest was antibody P215457 produced against PLC/PRF/5, which appeared to recognize epitopes present on two of three rat hepatoma cell lines as well as all four human HCC lines. The rat hepatoma cell lines were developed from tumors produced in vivo by chemical carcinogens (18, 19). In contrast, the PLC/PRF/5 cell line was derived from an HBsAg-positive chronic carrier with primary HCC. Tissue culture supernatant from PLC/PRF/5 has been shown to contain HBsAg and the cells contain from four to eight copies of hepatitis B virus (HBV)-DNA which is integrated into the cellular DNA (20–24). The Mahlavu cell line was also derived from a HBsAg-positive patient with HCC but apparently
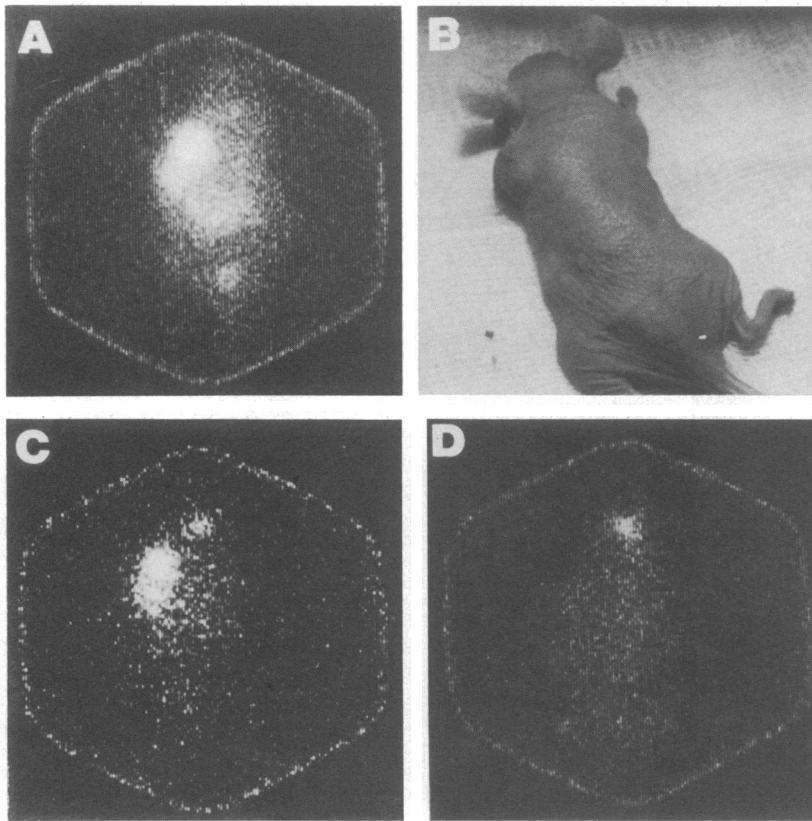
Figure 9. Radionuclide imaging of HCC PLC/PRF/5 tumor with Fab ^{131}I-P215457. Approximately 60 μCi of labeled antibody was injected into the tail vein. (*A*) Image created at 10 h after injection. (*B*) Size of nude mouse HCC tumor. (*C*) Image at 24 h showing precise localization of tumor and low uptake in other tissue and the blood pool. (*D*) Image at 24 h of a control nonhepatoma tumor demonstrating the specificity of P215457 Fab for HCC localization.

does not express HBV markers or contain HBV-DNA by molecular hybridization analysis (25). The SK-Hep 1 human hepatoma cell line was kindly provided by Dr. J. Fogh (Sloane-Kettering Institute, New York). Likewise, this cell line does not express HBsAg but does contain "liver-specific" protein on its cell surface (26). The cell line FOCUS developed in our laboratory was derived from a Greek patient with HBsAg-negative HCC. Human chromosomes have been identified by karotype and isoenzyme analysis. Both cell homogenate and spent culture medium are negative for HBsAg, hepatitis B core antigen, hepatitis B e antigen, and α-fetoprotein. The cells do, however, contain a single HBV integration site into the cellular DNA by molecular hybridization analysis (5). Thus the epitope recognized by P215457 is not species specific or associated with a presumed viral, nonviral, and/or chemically induced etiology of the hepatoma. However, this epitope appears to be relatively specific to transformed hepatocytes.

Further evidence for the specificity of three of the monoclonal antibodies for transformed hepatocytes is presented in Table II. We were unable to identify antigenic expression on 38 other human and nonhuman cell lines with the notable exception of a human colon adenocarcinoma cell line designated SW480. Further evidence for restriction of these antigenic determinants to transformed hepatocytes was the finding that such epitopes were absent on a variety of normal human tissues as well as breast, colon and pancreatic carcinoma metastatic to the liver. The hepatoma specificity demonstrated here by three of the anti-PLC/PRF/5 monoclonal antibodies has not been the experience of other investigators who have produced monoclonal antibodies to other human tumor cell lines. For example, antibodies directed toward melanoma (27,

28), breast adenocarcinoma (29, 30), colon adenocarcinoma (31–34), lung, and other tumors (35, 36) have been shown to cross-react with similar epitopes on normal and pathologic tissues as well as other malignant tumors and tumor cell lines. The reasons for the specificity of some of the anti-HCC monoclonal antibodies for transformed hepatocytes are not entirely clear but may be due in part to lack of enzyme treatment in the preparation of cells, methods of immunization, and the initial screening techniques.

Cell surface-labeling experiments with ^{125}I demonstrated two major proteins. Antibody P215457 precipitated a single protein of ~50,000 mol wt, whereas PM4E9917 precipitated a different protein of ~65,000 mol wt. Both were expressed on rat and human hepatoma cell lines. We were unable to precipitate similar proteins after in vitro labeling with [3H]glucosamine, suggesting that the proteins on which the monoclonal antibodies binding epitopes reside are not glucosamine-containing glycoproteins.

Previous studies have demonstrated under some circumstances the presence of soluble antigens secreted into the medium of colorectal as well as other cell lines (37, 38). Similarly, we searched for the presence of hepatoma-associated antigenic determinants in cell culture supernatants from human and rat hepatoma and other tumor cell lines using the newly constructed monoclonal RIAs. We employed methods, solid-phase supports, and assay designs similar to our previous studies with soluble antigens such as HBsAg, α-fetoprotein, human chorionic gonadotropin (hCG), and βhCG (9, 39–45). Fig. 4 demonstrates the presence of soluble antigen as shown by high binding activity in cell culture supernatants derived from HCC lines SK-Hep-1 and Mahlavu and rat hepatoma

HTC-41. To characterize further the kinetics of the antigen–antibody reaction, experiments were performed to assess the effect of temperature on binding activity as shown in Fig. 6. To our surprise binding activity was enhanced as a function of decreasing and not increasing temperature of incubation. The explanation for this finding is not clear but may be due in part to aggregation of antigen at 4°C, which may allow for a multivalent interaction of the antibody with a polydeterminant antigen in the RIA (6). Attempts were made to characterize the nature of the binding activity. As shown in Fig. 7, a protein of ~45,000 mol wt was isolated. It is noteworthy that this protein is similar but not identical in molecular weight to the protein precipitated from ^{125}I-labeled PLC/PRF/5 cells as shown in Fig. 3. Whether the soluble antigen represents a component of the identified cell surface protein (see Figs. 1 and 3), is secreted, or released into the medium awaits further investigation. However, it appears clear that human and rat hepatoma cell lines produce soluble hepatoma-associated antigens that may be detected by monoclonal RIAs and where the antibodies have been developed by immunization with intact cells. It will be of interest to determine if similar protein antigens are present in the blood of patients with primary hepatocellular carcinoma.

We took advantage of some of the physical properties of the anti-HCC monoclonal antibodies to perform in vivo radionuclide-imaging studies. In this regard we studied antibody P215457. Our initial studies focused on coupling P215457 to ^{99m}Tc and ^{111}In. We chose these reagents because, for example, ^{99m}Tc has an abundant proton emission at 140 keV, physical $t_{1/2}$ of 6 h, and no beta emission; such characteristics are well matched to present nuclear imaging instruments (46, 47). The first step involved the coupling of monoclonal antibody to DTPA (48, 49). The second step required an optimal dithionite concentration for reduction of ^{99m}TcO$_4^-$ and this was chelated to DPTA-coupled monoclonal antibody (50). Bound and free ^{99m}Tc was separated by staphylococcal A affinity chromatography followed by Sephadex G-25 chromatography. In using these procedures, 2–4 mCi of antibody conjugate was available for tail vein injection (100–200 μCi/mouse) into nude mice bearing tumors. Although uptake with both ^{99m}Tc and ^{111}In was evident in the PLC/PRF/5-induced tumor, substantial uptake was also evident in the liver and spleen (data not shown). Similar results have been observed by others using ^{111}In- and ^{125}I-labeled monoclonal antibody in guinea pigs bearing line 10 hepatocellular carcinomas (51). Because one of our goals is to have the capability to image within the liver, particularly in humans, we elected to abandon this approach.

In an attempt to improve the imaging of human hepatoma in nude mice, P215457 was radiolabeled with ^{131}I by the Iodogen technique to a specific activity varying between 14 and 20 μCi/μg. Animals with tumors were injected with 50–100 μCi in 200 μl via the tail vein; some of these results are shown in Fig. 8 and Table III. Although antibody uptake within the tumors and subsequent tumor visualization improved compared with the same antibody labeled with ^{99m}Tc and ^{111}In, the kinetics of tumor uptake with particular reference to the presence of radiolabeled antibody in the blood pool proved to be unsatisfactory. Thus, the slow disappearance of antibody from the blood pool relative to the tumor (animals were followed up to 96 h after tail vein injection) precluded any meaningful imaging results.

Stimulated by the experiments of Larson and colleagues

(52) on localization of ^{131}I-labeled Fab fragments in human melanoma, we prepared the Fab fragment of P215457 (kindly prepared by Dr. Jeffrey Mathis, Malvern, PA). Based on the initial binding characteristics of the Fab which showed a 10-fold reduction in binding activity to PLC/PRF/5 cells in suspension compared to the intact antibody, we would not have predicted that this fragment would be useful in tumor localization by nuclear imaging. In contrast, as shown in Table III and Fig. 9 there was rapid uptake within the tumor at 10 h (Fig. 9 A). Indeed, after rapid clearance of ^{125}I-P215457 Fab from the blood, there is clear localization of tumor by 24 h (Fig. 9 C); biodistribution experiments confirmed the imaging results (Table III). Particularly noteworthy was a ratio of ~9:1 when comparing uptake of antibody into tumor versus the liver. These results have led us to believe that the Fab fragment of P215457 may be a potent reagent for tumor localization within the liver in vivo.

There is little information regarding the properties of transformed hepatocytes. HCC appears to be one of the most common tumors in the world today. Indeed, projections from epidemiologic data on chronic hepatitis B carriers in Taiwan, suggests that a male has a 50% and female 20% chance of developing such tumors during their lifetime assuming the HBV infection was acquired at birth (1). In that it is estimated that there are 200–300 million chronic HBV carriers in the world (53), a need to understand the antigenic characteristics of this malignancy better and devise potential strategies for early immunodiagnosis clearly exists. In the present report we have demonstrated that monoclonal antibodies produced against transformed human hepatocytes may be useful in this regard. We are optimistic that such an approach will be helpful in the study of hepatocellular carcinoma in humans.

Acknowledgments

References

1. Beasley, R. P. 1982. Hepatitis B virus as the etiologic agent in hepatocellular carcinoma—epidemiologic considerations. Hepatology. 2:215–265.

2. Prevention of Liver Cancer. Report of a WHO Meeting. 1983. Technical Report Series 691. World Health Organization, Geneva.

3. Okuda, K., H. Obata, Y. Nakajima, T. Ohtsuki, N. Okazak, and K. Ohnishi. 1984. Prognosis of primary hepatocellular carcinoma. Hepatology. 4:35–145.

4. Primack, A., C. L. Vogel, S. K. Kyalwaz, K. Sebastias, J. L. Zieglar, R. Simon, and P. P. Anthony. 1975. A staging system for hepatocellular carcinoma: prognostic factors in Ugandan patients. Cancer. 35:1357–1364.

5. Lun, H., K. J. Isselbacher, J. R. Wands, H. M. Goodman, C. Shih, and A. Quaroni. 1984. Establishment and characterization of a new human hepatocellular cell line. In Vitro (Rockville). 20:493–504.

6. Wands, J. R., and V. R. Zurawski, Jr. 1981. High affinity monoclonal antibodies to hepatitis B surface antigen (HBsAg) produced by somatic cell hybrids. Gastroenterology. 80:225–232.

7. Ey, P. L., S. J. Prowse, and C. R. Jenkins. 1978. Isolation of pure IgG$_1$, IgG$_{2a}$, and IgG$_{2b}$ immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry. 15:429–436.

8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagents. *J. Biol. Chem.* 193:265–275.

9. Wands, J. R., R. I. Carlson, H. Schumaker, V. R. Zurawski, Jr., and K. J. Isselbacher. 1981. Immunodiagnosis of hepatitis B by epitope identification with high affinity IgM monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 78:1214–1218.

10. Kennet, R. H., T. J. McKern, and K. B. Brechtol, editors. 1980. Monoclonal antibodies. Hybridomas: a new dimension in biological analysis. Plenum Press, New York.

11. Soule, H. R., E. Linder, and T. S. Edgington. 1983. Membrane 126-kilodalton phosphoglycoprotein associated with human carcinomas identified by a hybridoma antibody to mammary carcinoma cells. *Proc. Natl. Acad. Sci. USA.* 80:1332–1336.

12. Soule, H. R., R. L. Sanford, and J. S. Butel. 1982. Detection of simian virus 40 surface-associated large tumor antigen by enzyme-catalyzed radioiodination. *Int. J. Cancer.* 29:337–344.

13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 277: 680–685.

14. Wands, J. R., R. R. Bruns, R. I. Carlson, A. Ware, J. R. Menitove, and K. J. Isselbacher. 1982. Monoclonal IgM radioimmunoassay for hepatitis B surface antigen: high binding activity in serum unreactive with conventional antibodies. *Proc. Natl. Acad. Sci. USA.* 79:629–635.

15. Brown, J. P., R. J. Woodbury, C. E. Hart, I. Hellstrom, and K. E. Hellstrom. 1981. Quantitative analysis of melanoma-associated antigen P97 in normal and neoplastic tissues. *Proc. Natl. Acad. Sci. USA.* 78:539–543.

16. Moriarity, D. M., N. Fox, D. P. Aden, J. R. Hoyer, and B. B. Knowles. 1983. Identification of human hepatoma-defined cell surface molecules. *Hybridoma.* 2:39–47.

17. Wiedman, K. H., L. K. Trejdosiewicz, and H. C. Thomas. 1983. Identification of membrane antigens expressed on primary liver cancer (PLC) cells but not normal untransformed hepatocytes. *Hepatology.* 3:865. (Abstr.)

18. Thompson, E. B., G. M. Tompkins, and J. I. Curran. 1966. Induction of tyrosine α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. *Biochemistry.* 56:296–303.

19. Richardson, V. I., A. H. Tashjian, Jr., and L. Levine. 1969. Establishment of a cloned strain of hepatoma cells which secrete albumin. *J. Cell Biol.* 40:236–247.

20. MacNab, G., J. Alexander, G. Lecatsas, E. M. Bey, and J. M. Urbanowicz. 1976. Hepatitis B surface antigen produced by a human hepatoma cell line. *Br. J. Cancer.* 34:509–515.

21. Alexander, J., G. MacNab, and S. Saunders. 1978. Studies on in vitro production of hepatitis B surface antigen by a human hepatoma cell line. *Perspect. Virol.* 10:103–120.

22. Stannard, L., and J. Alexander. 1977. Electron microscopy of HBsAg from a human hepatoma cell line. *Lancet.* II:713–714.

23. Marion, P., F. Slalzar, J. Alexander, and W. F. Robinson. 1980. State of hepatitis B viral DNA in a human hepatoma cell line. *J. Virol.* 33:795–805.

24. Dodd, R., T. Gouaux, and M. Bastians. 1978. Biosynthesis of HBsAg and serum proteins by a human hepatocellular carcinoma cell line. *In* Viral Hepatitis. G. Vyas, S. Cohen, and R. Schmidt, editors. Franklin Institute Press, Philadelphia. 698.

25. Prozesky, O. W., C. S. Brits, and W. O. K. Grabow. *In* Liver. E. J. Saunders, and J. Tarblanch, editors. Pitman Medical, London. 358–360.

26. Fogh, J., J. M. Fogh, and T. Orfeo. 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.* 59:221–226.

27. Dypold, W. G., K. O. Lloyd, L. J. C. Li, H. Ikeda, H. F. Oettgen, and J. O. Lloyd. 1980. Cell surface antigens of human malignant melanoma: definitions of six antigenic systems with mouse monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 77:6114–6118.

28. Mitchell, K. F., J. P. Fuhren, Z. Steplewski, and H. Koprowski. 1980. Biochemical characterization of human melanoma: dissection with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 77:7287–7291.

29. Colcher, D., P. H. Hand, M. Nuti, and J. Schlom. 1981. A spectrum of monoclonal antibodies reactive with human mammary tumor cells. *Proc. Natl. Acad. Sci. USA.* 78:3199–3203.

30. Taylor-Papadimitriou, J., J. A. Peterson, J. Arklie, J. Burchell, and R. L. Ceriani. 1981. Monoclonal antibodies to epithelium-specific component of the human milk fat globule membrane: production and reactions of cells in culture. *Int. J. Cancer.* 28:17–21.

31. Koprowski, H., Z. Steplewski, K. Mitchell, M. Herlyn, D. Herlyn, and J. P. Fuher. 1979. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somatic Cell Genet.* 5:957–971.

32. Herlyn, D., H. Koprowski, M. Herlyn, Z. Steplewski, D. Herlyn, and H. Koprowski. 1979. Colorectal carcinoma specific antigens; detection by means of monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 76:1438–1442.

33. Koprowski, H., H. Meehard, Z. Steplewski, and H. F. Sears. 1981. Specific antigen in serum of patients with colon carcinoma. *Science (Wash. DC).* 212:53–55.

34. Magnani, J. L., M. Brockhaus, D. F. Smith, V. Ginsburg, B. M. Blaszczyk, K. F. Mitchell, Z. Steplewski, and H. Koprowski. 1981. A monosialoganglioside is a monoclonal antibody-defined antigen of colon carcinoma. *Science (Wash. DC).* 212:55–56.

35. Mazauric, T., K. F. Mitchell, G. J. Letchworth III, H. Koprowski, and Z. Steplewski. 1982. Monoclonal antibody-defined human lung cell carcinoma surface protein antigen. *Cancer Res.* 42:150–154.

36. Old, L. J. 1981. Cancer immunology: the search for specificity. GHA Glowes Memorial lecture. *Cancer Res.* 41:361–375.

37. Herlyn, M., W. H. Clark, M. J. Mastrangelo, D. Guerry IV, D. E. Elder, D. LaRossa, R. Hamilton, E. Bondi, R. Tuthill, Z. Steplewski, and H. Koprowski. 1980. Specific immunoreactivity of hybridoma-secreted monoclonal anti-melanoma antibodies to cultured cells and freshly derived human cells. *Cancer Res.* 40:3602–3608.

38. Salter, D., B. Ballou, J. Relian, G. Levine, T. K. Hakala, and B. B. Knowles. 1982. Radioimmunodetection of tumors using monoclonal antibodies. *In* Hybridomas in Cancer Diagnosis and Treatment. M. S. Mitchell and H. F. Ottgen, editors. Raven Press, New York. 241–264.

39. Wands, J. R., R. A. Marciniak, K. J. Isselbacher, M. Varghese, G. Don, J. W. Halliday, and L. W. Powell. 1982. Demonstration of previously undetected hepatitis B viral determinants in an Australian aboriginal population by monoclonal anti-HBs antibody radioimmunoassay. *Lancet.* I:977–980.

40. Shouval, D., D. A. Shafritz, V. R. Zurawski, Jr., K. J. Isselbacher, and J. R. Wands. 1982. Immunotherapy in nude mice of human hepatoma using monoclonal antibodies against hepatitis B virus. *Nature (Lond.).* 298:567–569.

41. Bellet, D. H., J. R. Wands, K. J. Isselbacher, and C. Bouhoun. 1984. Serum alpha-fetoprotein in human disease: new perspectives by a monoclonal radioimmunoassay. *Proc. Natl. Acad. Sci. USA.* 81: 3869–3873.

42. Ben-Porath, E., J. R. Wands, M. Guria, and K. J. Isselbacher. 1984. Clinical significance of enhanced detection of hepatitis B surface antigen (HBsAg) by a monoclonal radioimmunoassay. *Hepatology.* 4: 932–938.

43. Wands, J. R., A. M. Wong, J. Shorey, R. Brown, R. A. Marciniak, and K. J. Isselbacher. 1984. Signature analysis of hepatitis B viral antigenic structure: Analysis by monoclonal radioimmunoassays. *Proc. Natl. Acad. Sci. USA.* 81:2237–2241.

44. Bilart, J. M., M. Ozturk, D. H. Bellet, M. Jolivet, H. Gras-Masse, F. Troalen, C. Bohoun, and J. R. Wands. 1985. Identification of epitopes associated with hCG and βhCG carboxy terminus by monoclonal antibodies produced against a synthetic peptide. *J. Immunol.* 134:457–464.

45. Bellet, D. H., M. Ozturk, J. R. Wands, J. M. Bidart, M. Assicot, F. Troalen, and C. Bohoun. Monoclonal antibodies directed

towards unique epitopes on human chorionic gonadotropin: an approach to the development of sensitive and specific radioimmunoassays. *In* Monoclonal Antibodies and New Trends in Immunoassays. Ch. A. Bizollon, editor. Elsevier Science Publishers, Amsterdam-New York-Oxford. 43–52.

46. Lebowitz, E., and P. Richards. 1974. Radionuclide generator systems. *Semin. Nucl. Med.* 4:257–268.

47. Benjamin, P. P., A. Rejali, and H. Friedell. 1970. Electrolytic complexation of ^{99m}Tc at constant current: Its applications in nuclear medicine. *J. Nucl. Med.* 11:147–154.

48. Krejcarek, G. E., and K. L. Tucker. 1977. Covalent attachment of chelating groups to macromolecules. *Biochem. Biophys. Res. Commun.* 77:581–585.

49. Khaw, B. A., J. T. Fallon, H. W. Strauss, and E. Haber. 1980. Myocardial infarct imaging of antibodies to canine cardiac myosin with indium-111-diethylenetriamine pentaacetic acid. *Science (Wash. DC).* 209:295–297.

50. Palk, C. H., R. P. Murphy, C. W. Eckelman, W. A. Volkert, and C. R. Reba. 1983. Optimization of the DTPA mixed-anhydride reaction with antibodies at low concentration. *J. Nucl. Med.* 24:532–536.

51. Bernhard, M. I., K. M. Hwang, K. A. Foon, A. M. Keenan, R. M. Kessler, J. M. Friche, D. J. Tallam, M. G. Hanna, L. Peters, and R. K. Oldham. 1983. Localization of ^{111}In and ^{125}I-labeled monoclonal antibody in guinea pigs bearing line 10 hepatocellular carcinoma tumors. *Cancer Res.* 43:4429–4433.

52. Larson, S. M., J. A. Carrasquillo, K. A. Krohn, J. P. Brown, R. W. McGuffin, J. M. Terens, M. M. Graham, L. D. Hill, P. L. Beaumice, K. E. Hellstrom, and I. Hellstrom. 1983. Localization of ^{131}I-labeled p97-specific Fab fragments in human melanoma as a basis for radiotherapy. *J. Clin. Invest.* 72:2101–2114.

53. Popper, H., M. A. Gerber, and S. N. Thung. 1982. The relationship of hepatocellular carcinoma to hepatitis B and related viruses in man and animals. *Hepatology.* 2:15–95.