Yonsei Medical Journal
Vol. 29, No. 1, 1988

Characterization of Tumor Specific Antigens on the Plasma Membrane Surface of Rat Hepatomas Induced by 3'-Me DAB and Identification of the Common Tumor Specific Antigens from Rat Hepatomas Induced by Different Chemical Hepatocarcinogens

Yoon Soo Kim[1], Kyung-Soo Hahm[2], Kyung Sup Kim[1] and Nam Jeen Lee[1]

Three different chemical carcinogens, 2-acetylaminofluorene (AAF), diethylnitrosamine (DENA), and 3'-methyl-4-dimethylaminoazobenzene (3'-Me DAB) were used to induce hepatomas in rats. Plasma membrane surface proteins of normal rat liver cells and rat hepatomas were extracted with 3M KCl. From the analysis of the proteins of normal rat liver and rat hepatoma induced by 3'-Me DAB by discontinuous polyacrylamide gel electrophoresis (Disc-PAGE), under nonreducing and nondenaturing conditions, polyacrylamide gel electrophoresis in the presence of SDS and 2-mercaptoethanol (SDS-PAGE), Sephadex G-200 gel permeation chromatography, DEAE-A50 ion-exchange chromatography and two-dimensional gel electrophoresis, at least three tumor specific antigens were identified. One had a molecular weigh of 66,000 (pI=6.79) while the other two had the same molecular weight, 73,000 but differed in their isoelectric points (7.58 and 7.81). For immunological analysis of tumor specific antigens, the absorbed antiserum was prepared. Plasma membrane surface proteins of rat hepatoma induced by 3'-Me DAB were used to obtain New Zealand White male rabbit antiserum. Rabbit antiserum was then reacted with the proteins isolated from the plasma membrane surface of normal rat liver and the absorbed antiserum reacting specifically with the tumor specific antigens derived by 3'-Me DAB was obtained. Using the absorbed antiserum, the immunoreactivities of plasma membrane surface proteins isolated from rat hepatomas induced by 3'-Me DAB, AAF, and DENA were compared by Ouchterlony double immunodiffusion analysis and immunoelectrophoresis. To characterize the proteins reacting to the absorbed antiserum, immunoglobulin G was separated from the absorbed antiserum and coupled to cyanogen bromide-activated Sepharose CL-4B. The isolated proteins from the plasma membrane surface proteins of 3'-Me DAB-induced hepatoma using this immunoaffinity chromatography had molecular weights of 66,000 and 73,000. The localization of these proteins on surface plasma membranes of rat hepatomas induced by 3'-Me DAB was confirmed by an immunofluorescence technique. The experimental results revealed the existence of cross-reacting common antigens on the plasma membrane surface of rat hepatomas induced by different hepatocarcinogens.

Key Words: Tumor specific antigen, hepatoma, hepatocarcinogen affinity chromatography

During malignant transformation, changes are known to occur in the proteins of the plasma membrane surface and cause the transformed cells to possess different physiological properties when compared to normal cells. Since Paul Ehrlich (1908) first reported that tumor cells could be recognized as foreign by the host cell because of this unusual property, many studies have been devoted to tumor specific antigens which are the new proteins appearing on the cell surface during malignant transformation. It is also known that different types of cells and carcinogens cause different changes in plasma membrane surface proteins. The plasma membrane of an animal infected with an RNA tumor virus displayed a new protein called a gross cell surface antigen (GCSA) and this antigen was immunogenic to the host

Received December 9, 1987
Accepted January 21, 1988

[1] Department of Biochemistry, Yonsei University College of Medicine, Seoul, Korea.
[2] Protein Chemistry Laboratory Genetic Engineering Center, Korea Advanced Institute of Science and Technology, Seoul, Korea.

This study was supported in part by grant 200-1146 from Korean Scientific Foundation.

Address reprint requests to Dr. Y S Kim, Department of Biochemistry, Yonsei University College of Medicine, Seoul, Korea 120-140

animal (Kurth *et al.* 1979).

There have also been studies on the tumor specific antigens induced by chemical carcinogens. Baldwin and Graves (1972) reported on a tumor specific antigen from the plasma membrane of an aminoazo dye (4-dimethylaminoazobenzene; DAB)-induced rat hepatoma. They used limited papain digestion to solubilize plasma membrane fractions isolated from the cells of a DAB-induced rat hepatoma and isolated a tumor specific antigen capable of neutralizing antibodies in a tumor-immune sera by DEAE cellulose chromatography. Natori *et al.* (1977) used Nonidet P-40 to isolate a fibrosarcoma specific antigen from mice fibrosarcoma induced with methylcholanthrene and reported that the molecular weight was 70,000.

On the other hand, Pellis *et al.* (1976) obtained a tumor specific transplantation antigen (TSTA) from 3M KCl extracts of 3-methylcholanthrene- induced murine fibrosarcoma after treating the tumor cells with trypsin and purified by ammonium sulfate fractionation and polyacrylamide gel electrophoresis (PAGE). They isolated two components showing TSTA activity which had isoelectric points of 5.05 and 6.90, and estimated molecular weights of 40,000 and 75,000, respectively.

Since the tumor specific antigens described above were obtained by using either a proteolytic enzyme or nonpolar detergent, these antigens may be a part of a molecule instead of being the complete one. Recently, a method using 3M KCl for isolating the complete form of an antigen from the plasma membrane surface was reported by Stevens *et al.* (1981), and by using this method, three tumor specific antigens were identified (Lim *et al.* 1982) on the surface of the plasma membrane of rat hepatoma induced by 3'-methyl-4-dimethyl-aminoazobenzene (3'-Me DAB).

The studies described herein were initiated to purify the tumor specific antigens identified from the plasma membrane surface of a rat hepatoma induced by 3'-Me DAB, to characterize their molecular properties, and to see whether common tumor specific antigens are formed when hepatomas were induced by other known hepatocarcinogens such as 2-acetylaminofluorene (AAF) and diethylnitrosamine (DENA).

MATERIALS AND METHODS

Induction of Tumors: Liver tumors were induced in male Spargue Dawley rats (about 80g) by the protocols shown in Table 1 using 3'-Me DAB (Eastmman Kodak Co.), AAF (Sigma Chemical Co.), and DENA (Sigma Chemical Co.). The first group was given 0.02% AAF (Peraino *et al.* 1971) in a synthetic diet (Lim *et al.* 1982) ad libitum for 8 weeks followd by a 0.05% phenobarbital containing diet, and a liver tumor was observed after 48 weeks of feeding. The second group was given DENA (Lijisky and Taylor, 1978) in the drinking water (0.007%) for 30 weeks (100ml/day/cage of 5 rats for 5 days a week) ad libitum. Liver tumors were induced in the third group by feeding with 0.06% 3'-Me DAB in a diet for 12 weeks followed by a control diet ad libitum for 4 weeks (Lim *et al.* 1982). For each treatment group, an appropriate control group was maintained to obtain normal livers.

Table 1. Rat liver tumor induction by hepatocarcinogens

Carcinogen	Method of Administration	Duration for Tumor Induction
2-Acetylamino-fluorene (AAF)	0.02% AAF in a diet for 8 wks followed by 0.05% phenobarbital containing diet.	48 wks
Diethylnitrosa-mine (DENA)	0.007% in the drinking water 10 ml/day/cage of 5 rats for 5 days a week.	30 wks
3'-Methyl-4-di-methylaminoa-zobenzene (3'-Me DAB)	0.06% in a diet for 12 wks followecd by control diet.	16 wks

Extraction of Plasma Membrane Surface Proteins: Extraction of surface proteins from the plasma membrane of liver cells of normal rats and bearing liver tumors was carried out using 3M KCl essentially according to the procedure of Stevens *et al.* (1981). The liver was removed from the rat under ether anesthesia after perfusing it with physiological saline containing 0.5mM $CaCl_2$.

Minced liver was washed by centrifugation at 5,000 × g for 30 min three times with phosphate buffered saline (PBS), pH 7.4 before extracting surface proteins with 3M KCl in PBS, pH 7.4 (2ml per g of tissue) for 18 hours at 4°C with constant stirring. The mixture was centrifuged (Sorvall RC-5B Refrigerated Superspeed Centrifuge; Du Pont Instruments) at 20,000×g for 30 min followed by centrifugation at 110,000 × g for 90 min (L2-65B Ultracentrifuge; Beckman). The supernatant was dialyzed against PBS, pH 7.4 followed by 1mM EDTA-water, pH 7.4. The dialysate was centrifuged at 110,000 × g for 90 min and the clear supernatant was sterilized by passing through a membrane filter (0.45 μm; Gelman). The

solution was divided into 1.0ml aliquots and stored frozen at -70°C. Protein concentrations were determined by the modified micromethod of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Preparation of Immune Sera: New Zealand White male rabbits (2kg) were used to obtain immune sera. The rabbits were immunized with plalsma membrane surface proteins isolated from the liver tumor cells induced by 3'-Me DAB (500 μg protein per 2 ml of complete Freund adjuvant) followed by a booster injection of 300 μg protein in 1 ml of incomplete Freund adjuvant two weeks after the first injection. Serum was separated two weeks after the booster shot. Serum from normal rabbits was also separated after an 18 hr fast and used as the control serum. The rabbit antiserum was reacted with control proteins isolated from the plasma membrane of normal rat liver (50 mg proteins per ml of antiserum) at 37°C for 1 hr and the absorbed serum specific for tumor associated antigen was separated by centrifuging at 8000×g for 10 min. The clear supernatant wass passed through 0.45 μm membrane filter and stored at −70°C in 1 ml aliquots.

Immunofluorescence Analysis: Localization of tumor specific antigens on the plasma membrane surface of rat hepatoma induced by 3'-Me DAB was confirmed by the immunofluorescent technique. Mono-cellular suspensions of normal rat liver and rat hepatic tumor induced by 3'-Me DAB were prepared by the enzymatic method using hyaluronidase according to the procedure described by Howard *et al.* (1967). The cell suspension in calcium free Hanks' solution were semared on the slides and the absorbed rabbit antiserum (1:10 diluted) specific for 3'-Me DAB-induced hepatoma was added. After incubating the slides at 40°C for 30 min, goat anti-rabbit IgG-FITC (1:80 diluted) was added and the slides were incubated at 40°C for 30 min. The slides were then stained with Evans blue, were viewed under a UV lamp and picture were taken.

Electrophoresis: Discontinuous polyacrylamide gel electrophoresis (Disc-PAGE) under nonreducing and nondenaturing conditions (7% separating gel, 4% stacking gel) and PAGE containing 0.1% sodium dodecyl sulfate (SDS-PAGE) were carried out according to the procedure of Hahm *et al.* (1983) in order to identify and characterize the tumor specific antigens. Immunoelectrophoresis (Graber and Williams, 1953) using the absorbed antiserum specific for 3'-Me DAB induced rat hepatoma was performed on the test surface proteins of the plasma membranes of hepatomas induced by 3'-Me DAB, AAF, and DENA. Two dimensional gel electrophoresis (O'Farrell, 1975) was performed in order to identify the tumor specific antigens. Plasma membrane surface proteins of normal rat liver and rat hepatima induced by 3'-Me DAB were subjected to first dimensional isoelectric focusing in tube gels under denaturing and reducing conditions. Focusing was done in the pH range of 4 to 9 using 1 mg protein each. Upon completion of isoelectric focusing, gel were placed on top of 10% polyacrylamide slab gels containing 0.1% SDS and second dimensional electrophoresis was performed in the vertical electrophoresis unit (Model 2001; LKB Bromma).

Double Immunodiffusion: Ouchterlony double immunodiffusion analysis (Ouchterlony, 1949) in 1% agarose (Agarose-M, LKB Bromma) was used to compare the plasma membrane surface proteins isolated from liver tumor cells induced by 3'-Me DAB, AAF, and DENA.

Preparation of immunoaffinity column for identification of the tumor specific antigens: Immunoglobulin G was separated from the absorbed antiserum using Protein A-Sepharpse 6MB (Ery *et al.* 1978). Purified IgG in 0.5M NaCl, 0.1M sodium bicarbonate buffer pH 8.5 was coupled to cyanogen bromide-activated Separose CL-4B at the ratio of 7 mg/ml of packed beads (Choo *et al.* 1981). This column (1×10cm) was pre-eluted with 50ml M NaCl, 0.1M Tris-HCl buffer pH 8.6. The cell surface proteins from hepatoma induced by 3'-Me DAB were applied to the immunoaffinity column at a rate of 15 drops/min. After 15mg of the protein sample was loaded, the column was washed with 0.5M NaCl, 0.1M Tris-HCl pH 8.0 until the absorbance of the elulents at 280nm was less than 0.02. The column was then eluted with 150mM diethylamine pH 11.5. One ml fractions were collected, their absorbance measured 280nm and then they were immediately neutralized with 0.5M Tris-HCl buffer pH 7.6. Fractions of the protein peak were pooled and analyzed by SDS-PAGE.

Purification of Tumor Specific Antigens: Tumor specific antigens associated with rat hepatoma induced with 3'-Me DAB were differentiated and separated by gel permeation column chromatography using Sephadex G-200 (Pharmacia Fine Chemicals) and ion-exchange chromatography on a column of DEAE-Sephadex A 50 (Pharmacia Fine Chemicals).

RESULTS

Plasma membrane surface proteins were isolated from the livers of rats fed the control diet and the 3'-Me DAB containing diet at 3,6,8,10,12,14 and 18

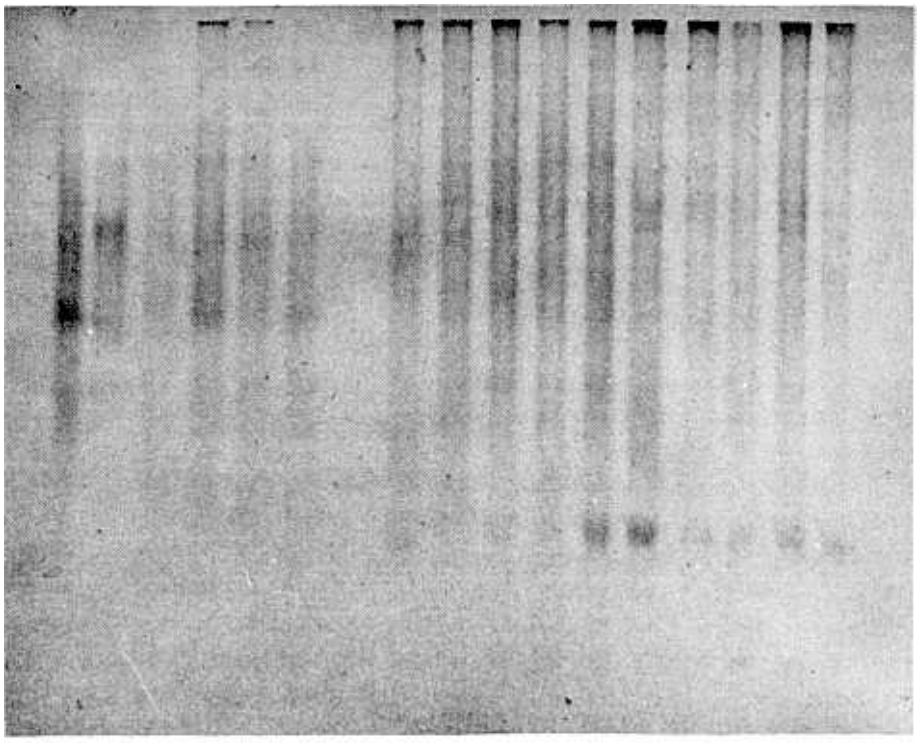


Fig. 1. 7% Disc PAGE analysis of plasma membrance surface proteins of rat liver cells. Lanes 1 through 6; plasma membrane surface proteins (100 μg each) extracted from normal rat liver cells isolated at 3, 6, 8, 10, 12 and 14 weeks of feeding control diet. Lanes 7 through 16; plasma membrane surface proteins (100 μg each) extracted from liver cells of rats sacrificed at 3, 6, 8, 10, 12, 12 + 4, 12 + 6, 14, 14 + 4, 14 + 4 weeks of feeding 3'-Me DAB containing diet.*

** The numbers indicate the duration of feeding 3'-Me DAB containing diet followed by the duration of feeding control diet.*

weeks of feeding, and analyzed by polyacrylamide slab gel electrophoresis. When the surface proteins were analyzed by 7% Disc-PAGE (Fig. 1), a new protein band (R_f=0.62) was identified from the surface proteins of tumor bearing rat liver. The band could not be seen in control protein samples (Fig. 1, Lanes 1 through 6), but it started appearing in the surface proteins obtained from rats fed with 3'-Me DAB even before the rat livers showed solid tumors (Lane 11). However, when the rats developed liver tumors, it was shown that the new protein band became heavy and more apparent (Fig. 1, Lane 11 through 16).

When the proteins were analyzed by 7% SDS-PAGE (Fig. 2), the differences between the control proteins (from normal rat livers; lanes 1 through 6, from livers of rats fed with 3'-Me DAB containing diet before developing tumors; lanes 7 throughh 10) and the plasma membrane surface proteins of rat hepatoma (lanes 11 through 16) could be recognized as several protein bands of which two protein bands (MW of approximately 66,000 and 73,000) were most apparent. Lane 17 shows the bands of molecular weight standards representing BSA (MW, 66,000) and ovalbumin (OVA; MW, 45,000), respectively.

To determine whether the antigens recognized by the absorbed specific antiserum for 3'-Me DAB are indeed located on the surface of the plasma membrane, the hepatomas induced by 3'-Me DAB were reacted with the absorbed antiserum, and the binding was monitored with goat anti-rabbit IgG conjugated to fluorescein. As is evident in Fig. 3, the plasma membrane of hepatoma (Fig. 3,B) stains very brightly, suggesting that these antigens are indeed membrane surface-associated antigens.

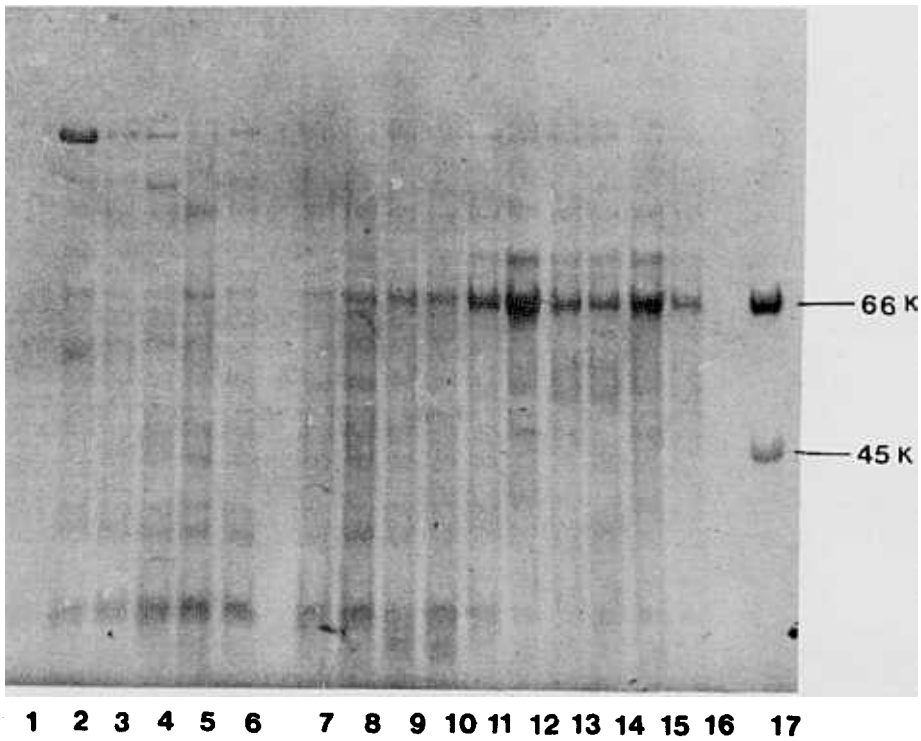


Fig. 2. 7% SDS-PAGE analysis of plasma membrane surface proteins of rat liver cells. Lanes 1 through 16 contain idential samples as in Fig. 1 except that land 17 shows the molecular weight standards; BSA, 66,000 and OVA, 45,000.

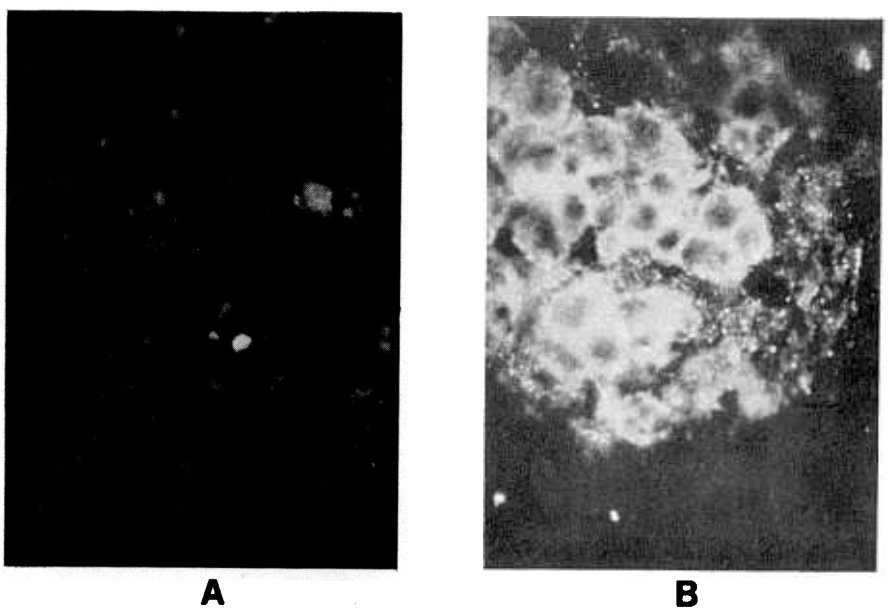


Fig. 3. Immunoflourescence staining patterns of normal rat hepatocyte (A) and rat hepatoma (B) induced by 3'-Me DAB (× 100). The plasma membrane of hepatoma (B) stains very brightly showing that the antigens recognized by the absorbed rabbit antiserum specific for 3'-Me DAB are on the membrane.

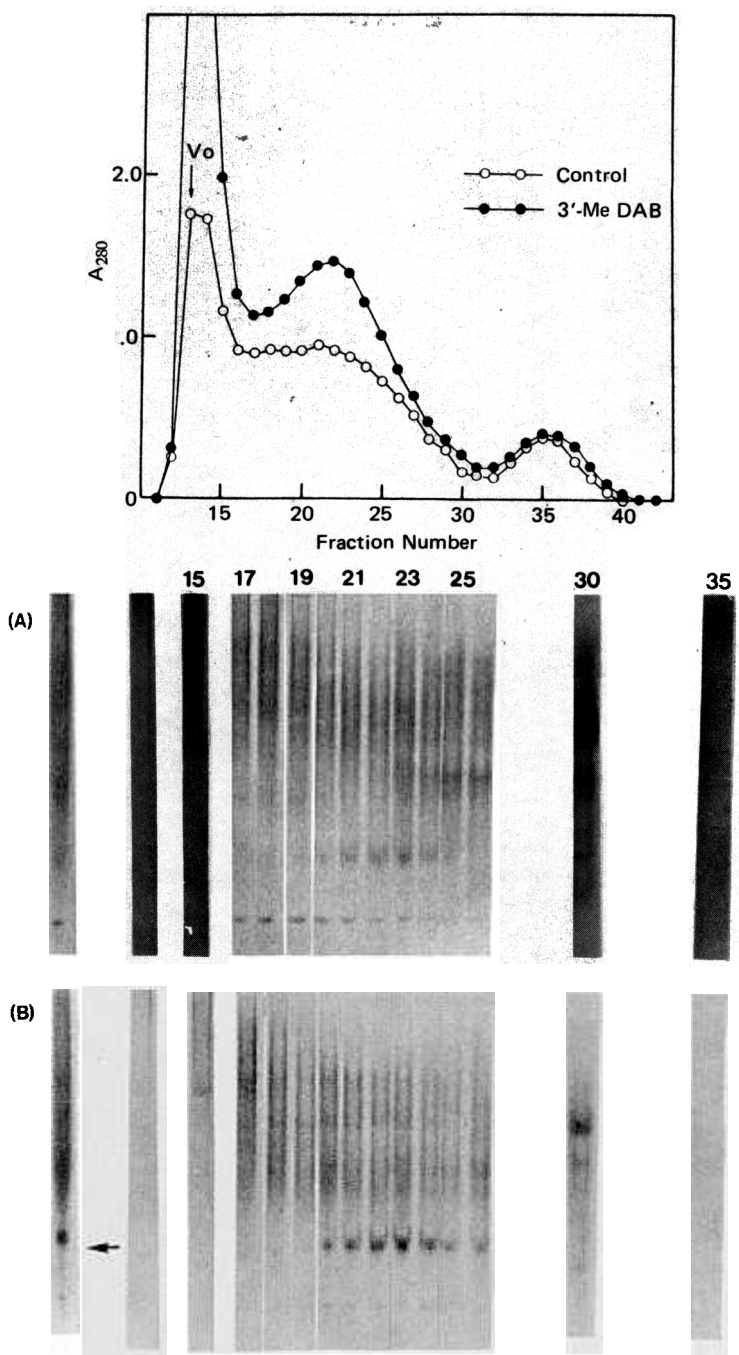
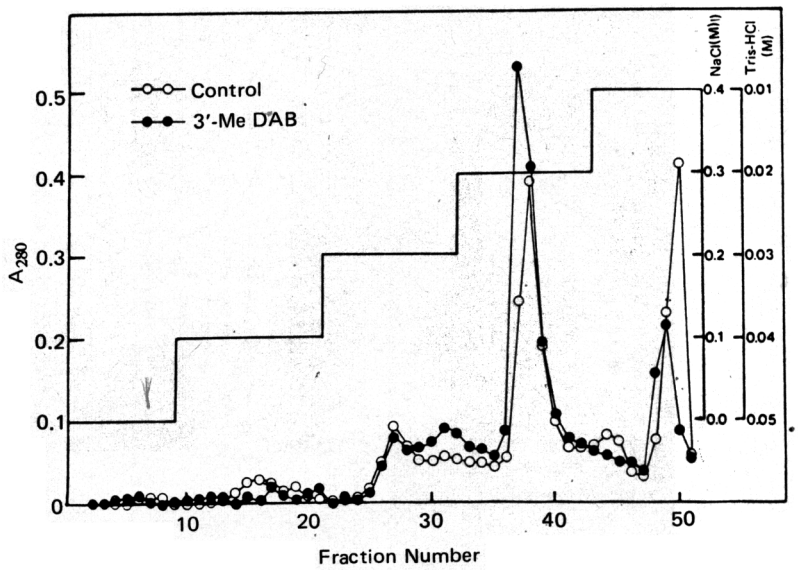


Fig. 4. Upper panel: Sephadex G-200 gel permeation column (0.9 × 56 cm) chromatography of the plasma membrane surface proteins (50mg) separated from the normal rat liver cells (o-o) and rat hepatoma induced by 3'-Me DAB (•–•). The elution buffer was 1 mM EDTA-water, pH 7.4 and V_o indicates the void volume of the column. Lower panel: Analysis of column fractions by 7% Disc-PAGE (A; control, B; 3'-MeDAB-induced hepatoma). Each lane represents the faction directly above in the elution profile except that the far left land shows each proteins before applying to the column. The tumor specific surface protein is indicated by an arrow.



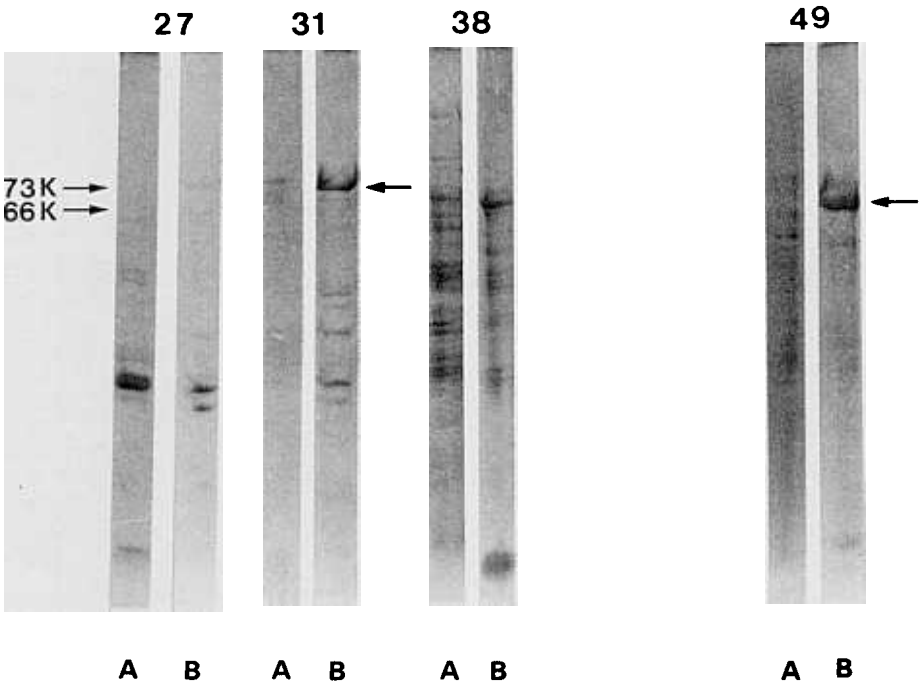


Fig. 5. Analysis of the plasma membrane surface proteins by ion-exchange chromatography on DEAE Sephadex A-50 and SDS-PAGE. After gel filtration (Fig. 3), fractions (16-26ml) containing tumor specific antigen (or corresponding fractions in the case of control proteins) were pooled, concentrated and applied to the column (0.9 × 56 cm) of DEAE A-50. Upper panel shows the absorbancy profile of plasma membrane surface proteins of normal rat liver cells (o–o) and of rat hepatoma (•–•) eluted with step gradient formed by 0-0.5 M NaCl in 0.05 M Tris-Cl buffer, pH 7.4. Each fraction contained 3.0 ml. Lower panel shows 10% SDS-PAGE analysis of the column eluents. The numbers above gel lanes refer to the fraction number. A refers to the fraction of control proteins and B for proteins of 3'Me DAB induced hepatoma.

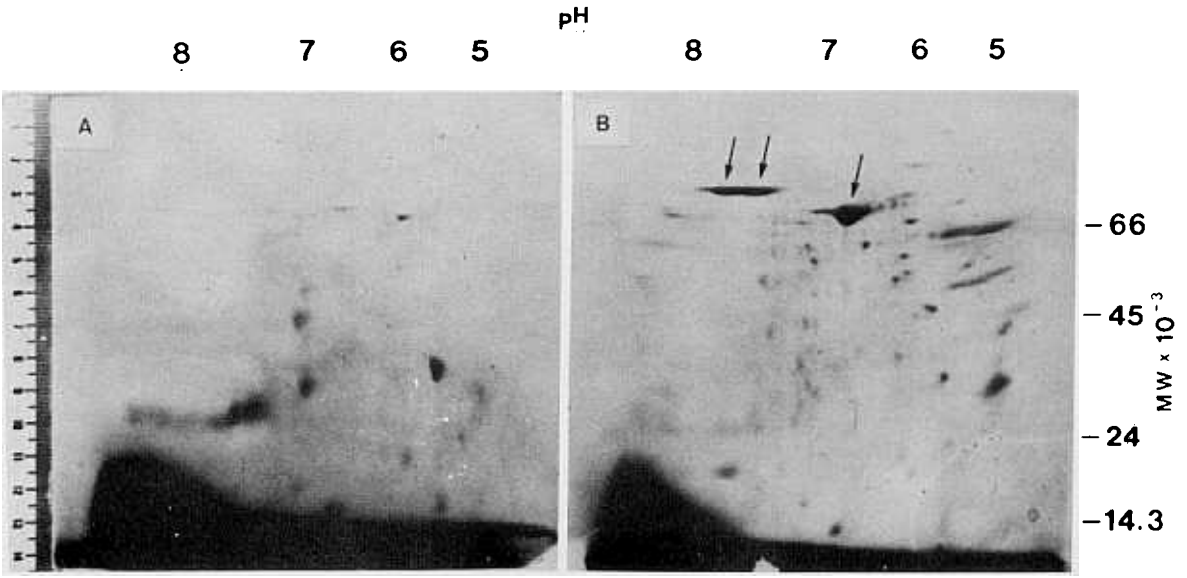


Fig. 6. Analysis by two dimensional gel electrophoresis of plasma membrane surface proteins isolated from normal rat liver cells (Panel A) and 3'-Me DAB induced rat hepatoma (Panel B). The major tumor specific proteins are indicated by arrows.

Fig. 4 compared the plasma membrane surface proteins of normal rat liver and 3'-Me DAB induced rat hepatoma by the elution on a column of Sephadex G-200 and showed the analysis of its fractions by 7% Disc-PAGE. The upper panel shows the elution profiles of the control proteins (-o-o-) and the proteins of 3'-Me DAB induced rat hepatoma (-•-•-). The result shows an almost identical elution pattern for both proteins except an increased protein peak for 3'-Me DAB induced hepatoma eluuting in the inclusion volumn (19-26ml). When analyzed were by 7% Disc-PAGE (Fig. 4, lower panel), the tumor associated protein was found to be localized in this peak area (19-26ml). The fractions of both proteins corresponding to this peak were pooled, concentrated, loaded on to a column of DEAE Sephadex A-50, and eluted with a step gradient formed with 0 to 0.4 M NaCl in 0.05 M Tris-Cl buffer, pH 8.3. The result (Fig. 5, upper panel) also showed nearly identical elution patterns for both proteins. However, when the column fractions were analyzed by 10% SDS-PAGE (Fig. 5, lower panel), the tumor specific antigen of 73K dalton was located in the peak including the fraction number 31, and the tumor specific antigen of 66K dalton was found in the last peak eluted with 0.4 M NaCl.

The plasma membrane surface proteins of normal rat liver and 3'-Me DAB induced rat hepatoma were also analyzed by two dimensional gel electrophoresis. From the result (Fig. 6), at least three tumor specific antigens which were not present in control proteins (Fig. 6, left panel) were identified (Fig. 6, right panel). They are a protein of 66K dalton (pI of 6.79) and two proteins of similar molecular weight (73K dalton) differing in their isoelectric pH (7.58 and 7.81).

From the above results, it is clearly demonstrated that there are at least three tumor specific antigens on the plasma membrane surface of rat hepatoma induced by the azo dye carcinogen, 3'-Me DAB.

With the above information, the following experiments were carried out to see whether any identical tumor specific antigens could be identified on the plasma membrane surface of rat liver tumors induced by various other known chemical hepatocarcingens.

Fig. 7 shows the result of Ouchterlony double immunodiffusion analysis using the absorbed antiserum specific for 3'-Me DAB-induced rat hepatoma associated plasma membrane surface proteins. The result indicates that there are common tumor specific antigens on the plasma membrane surfaces of rat hepatomas induced by 3'-Me DAB and DENA judging by the continuing precipitin band. A similar result was also obtained (date not shown) with AAF indicating that all of the above three carcinogens cause the synthesis of common tumor specific antigen(s).

The immunoreactivities of the plasma membrane surface proteins of rat hepatomas induced by 3'-Me DAB, DENA, and AAF where also compared by im-

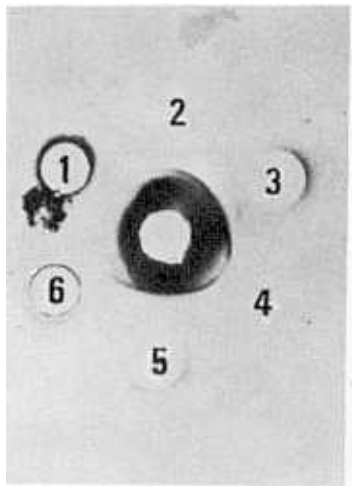


Fig. 7. Ouchterlony double immunodiffusion analysis of plasma membrane surface proteins isolated from rat liver cells. The absorbed rabbit antiserum (10μl) specific for 3'-MeDAB-induced hepatoma allowed to react by diffusion for 18 hrs at 25°C with the plasma membrane surface proteins (100μg) of rat liver cells in the outer cells. Well No. 1; normal rat liver cells, wells No. 2, 3, 5; 3'-Me DAB-induced rat hepatoma, Wells No. 4 and 6; DENA-induced rat hepatoma.

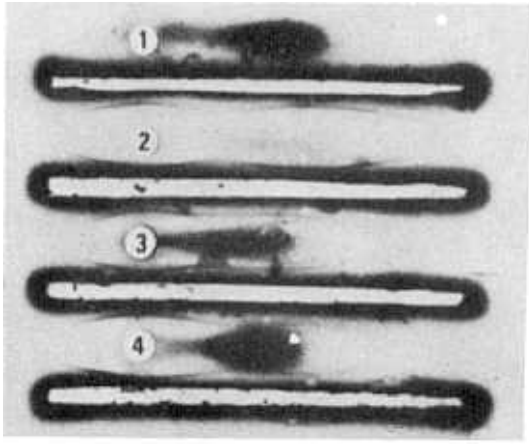


Fig. 8. Immunoelectrophoresis pattern using the absorbed rabbit antiserum specific for 3'-Me DAB-induced hepatoma and plasma membrane surface proteins isolated from various carcinogen induced rat hepatoma. Proteins (150 μg each) in wells (well No. 1; control protein from normal rat liver cells, well No. 2; 3'-Me DAB-induced rat hepatoma, well No. 3; AAF-induced rat hepatoma, and well No. 4; DENA-induced rat hepatoma) were electrophoresed for 2 hrs at 100 V before the diffusion at 25°C for 18 hrs with the absorbed antiserum (100 μl) in troughs.

munoelectrophoresis using the absorbed antiserum reacting specifically with the tumor specific antigens on the plasma membrane surface of rat hepatoma induced by 3'-Me DAB. The results (Fig. 8) show that the absorbed specific antiserum for 3'-Me DAB associated antigens recognized in the same way the antigens from the plasma membrane surface proteins of rat hepatomas induced by both AAF and DENA. When analyzed by 7% PAGE, the plasma membrane surface proteins of rat hepatomas induced by DENA and AAF also showed the protein bands characteristic for the rat hepatoma induced by 3'-Me DAB (Data not shown).

IgG separated from absorbed antiserum was coupled to cyanogen bromide-activated Sepharose CL-4B. The protein content of the bead indicated that 96% of the antibody was coupled. Tumor specific proteins

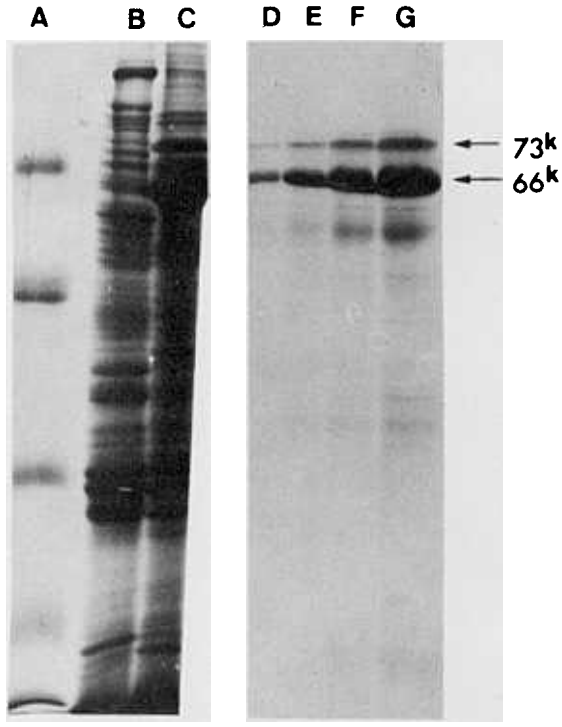


Fig. 9. Analysis of hepatoma-specific cell plasma membrain surface antigens eluted from immunoaffinity ohromatography Lane A; MW standards, BSA (MW=66,000), OVA (MW=45,000), trypsinogen (MW=24,000), and lysozyme (MW=14,300), lane B, C; total cell plasma membrane surface proteins (100μg) of normal rat liver (B) and rat hepatoma (C), lane D,E,F,G; hepatoma specific cell surface antigens (10, 25, 50, 100μg each) eluted from immunoaffinity chromatography.

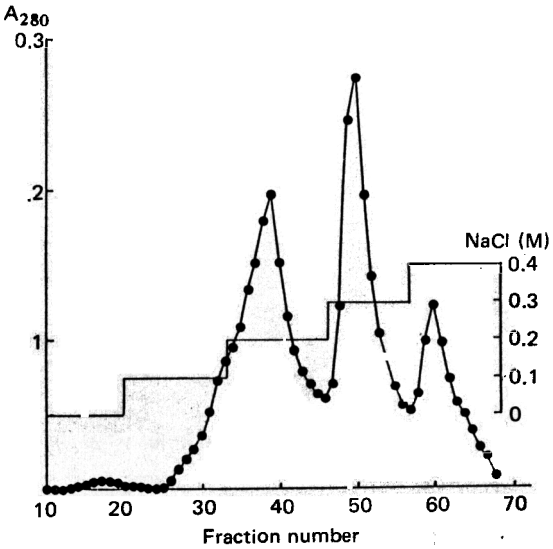


Fig. 10. DEAE Sephadex A-50 column chromatography. A 3ml sample from immunoaffinity column was applied on a column (2.5 × 10 cm) of DEAE Sephadex A-50 and eluted with a step gradient (–) formed by 0-0.4M NaCl in 0.05M Tris buffer, pH 8.3.

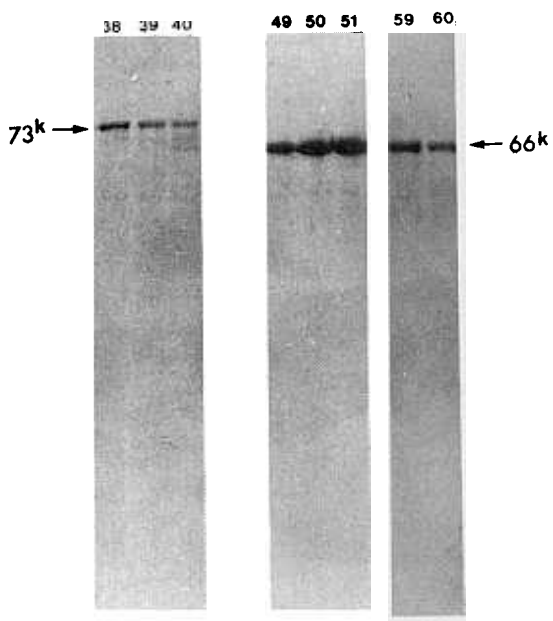


Fig. 11. SDS polyacrylamide gel (10%) electrophoretic patterns of proteins of various fractions (38, 39, 40, 49, 50, 51, 59, 60) eluted from DEAE Sephadex A-50.

separated by this immunoaffinity chromatography showed two major bands at the site of MW 73,000 and 66,000 on the SDS-PAGE (Fig. 9). These eluted proteins were further purified by DEAE-Sepharose A50 ion-exchange chromatography (Fig. 10) and analyzed by SDS-PAGE (Fig. 11). The results were identical to Fig. 5.

From these results, it is concluded that there are common tumor specific antigens on the plasma membrane surfaces of rat hepatomas induced by different chemical carcinogens, 3'-Me DAB, DENA, and AAF.

DISCUSSION

Since butter yellow (DAB) was reported to have a carcinogenic action by Kiosita (1936), many studies have reported on the morphological changes of rat liver during carcinogenesis induced by DAB (Orr 1940; Edwards and White 1942; Opie 1944) and the similarities of liver tumors induced by DAB and its derivatives (Cortell 1947; Miller and Miller 1948). Cunningham *et al.* (1950) studied the early changes in the liver cellls by 3'-Me DAB and Richardson and Nachtnebel (1951) reported on the frequencies of tumor incidences and metastasis. Farber (1956) compared early histological changes in rat livers induced by ethionine, AAF and 3'-Me DAB and reported that they induced similar histological changes and thus caused similar metabolic effects. It is also known (Miller 1970) that AAF, DENA, and 3'-Me DAB are the same type of chemical carcinogens in the sense that they all create carcinogenic electrophilic reactants (ultimate carcinogens) when metabolized. AAF metabolizes to yield the more carcinogenic N-hydroxy-2-acetylaminofluorene and DENA yields the carcinogenic electrophile, $C_2H_5^+$. Metabolism of 3'-Me DAB is also known to involve N-demethylation and N- and ring hydroxylation followed by conjugation with a sulfate moiety to yielld a strong electrophilic ester.

Miller and Miller (1947) first reported that DAB, when metabolized, formed a covalent linkage with the proteins of rat hepatocytes. It was also reported (Sorof *et al.* 1970; Sorof and Young 1973) that the metabolic product of 3'-Me DAB induced the h2-5s azoprotein with a specific protein in rat liver whose molecular weight was 60,000-80,000. The specificity and the function of the protein binding with the metabolities of the carcinogens are shown by Mainigi and Sorof by presenting evidence of the receptor protein which carries the metabolities of 3'-Me DAB. It was also reported by Patel (1979 and 1981) that 3'-

Me DAB acts as a kind of inhibitor for nuclear RNA processing, and thus causing distortion of RNA processing and resulting in a hepatoms. Bebawi *et al.* (1970) have shown that 3'-Me DAB is a more potent carcinogen than other structurally related DAB's.

At present, malignant tumors are thought to be developed in six successive steps (Kotin, 1976). They are 1. reaction between chemical carcinogens and nucleic acids, 2. effective repairing, 3. production of abnormal proteins induced by alterations of the genome, 4. progression of malignant changes, 5. formation of a cell mass in order to develop a malignant tumor from transformed cells, and 6. the growh of the tumor after the formation of a mass.

The presence of tumor specific antigens on the plasma membrane of DAB-induced hepatoma (D23) was first reported by Baldwin *et al.* (1971). They identified tumor specific antigens on the surface of a hepatoma by the indirect immunofluorescent antibody technique and studied the changes of the protein antigens during malignant transformation. This tumor specific antigen induced by DAB was separated by DEAE-cellulose column chromatography and gradient centrifugation after digesting the hepatoma with papain and the molecular weight was reported to be about 55,000 (Baldwin and Glaves, 1971; Baldwin *et al.* 1973). This protein is smaller than the DENA-induced guinea-pig hepatoma specific antigen of MW 75,000-150,000 extracted with 3M KCl (Meltzer *et al.* 1971). The tumor cell antigen isolated from papain digestion may not be an intact molecule. Pellis *et al.* (1976) separated two tumor specific antigens (MW 40,000 and 75,000) from the 3M KCl extract of methylcholanthrene-induced fibrosarcoma in C3H/HeJ mice after 0.25% trypsin digestion. It has been suggested that in order to isolate plasma membrane surface proteins in their intact molecular forms, proteolytic enzymes or detergents should be avoided.

In the present sudy, tumor specific plasma membrane surface proteins induced by 3'-Me DAB, AAF, and DENA were isolated using 3M KCl, and analyzed by the combined techniques of Disc PAGE, SDS-PAGE, and two-dimensional gel electrophoresis. It was identified that at least three tumor specific proteins of which molecular weight are 66 kdal (pI=6.79) and 73 kdal (pI=7.58 and 7.81), are newly expressed. The tumor specificity of these proteins on 3'-Me DAB induced hepatoma were confirmed by double immunodiffusion, immunoelectrophoresis, and immunoaffinity chromatography. The localization of these proteins was confirmed by the immunofluorescent technique. These results also provide evidence that there are common tumor specific antigens on the plasma membrane surface of rat hepatomas induced by different chemical carcinogens.

The 3'-Me DAB-induced tumor specific antigens have been partially purified by gel filtration chromatography and ion-exchange chromatography, and the efforts to characterize the molecular properties of these proteins and to elucidate the biological functions of these proteins are being made in this laboratory.

REFERENCES

Baldwin RW, Baker CR, Embleton MJ, Glaves D, Moore M, Pimm MV: Demonstration of cell-surface antigens on chemically induced tumors. *Ann N Y Acad Sci 177:268-278, 1971*

Baldwin RW, Graves D: Solubilization of tumor specific antigen from plasma membrane of an aminoazo dye-induced rat hepatoma. *Clin Exp Immunol 11:51-56, 1972*

Baldwin RW, Harris JR, Price MR: Fractionation of plasma membrane-associated tumor specific antigen from an aminoazo dye-induced rat hepatoma. *Int J Cancer 11:385-397, 1973*

Bebawi GM, Kim YS, Labooy JP: A study of the carcinogenesis of a series of structurally related 4-dimethylaminoazobenzenes. *Cancer Res 30:1520-1524, 1970*

Choo KH, Jennings IG, Cotton GH: Comparative studies of four monoclonal antibodies to phenylalanine hydroxylase exhibiting different properties with respect to substrate dependence, species-specificity and range of effects on enzyme activity. *Biochem J 199:527, 1981*

Cortell R: The production of tumors in the livers of rats fed 3'-methyl-p-dimethylaminoazobenzene. *Cancer Res 7:158-161, 1947*

Cunningham L, Griffin AC, Luck JM: Effect of a carcinogenic azodye on liver cell structure. Isolation of nuclei and cytoplasmic granules. *Cancer Res 10:194-199, 1950*

Edwards JE, White J: Pathologic changes with special reference to pigmentation and classification of hepatic tumors in rats fed p-dimethylaminoazobenzene (Butter Yellow). *J Natl Cancer Inst 2:157-183, 1942*

Ehrlich P: *Uber den jetzigen Stand der Karzinomoforschung.* In: The collected papers of Paul Ehrlich. Vol 11., Pergamon Press, London, *1908, 550*

Ey PL, Prowse SJ, Jenkin CR: Isolation of pure IgG_1, IgG_2a and IgG_2b immunoglobulins from mouse serum using Protein A-Sepharose. *Immunochemistry 15:429, 1978*

Farbe E: Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene, and 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res 16:142-148, 1956*

Graber P, Williams CA Jr: *Biochem Biophys Acta 10:193, 1953*

Hahm KS, Tikkanen MJ, Dargar R, Colé TG, Davis JM, Schonfeld G: Limited proteolysis selectively destroys epitopes on apolipoprotein B in low density lipoproteins. *J Lipid Res 24:887, 1983*

Haward RB, Christensen AK, Gibbs FA, Pesch LA: The enzymatic preparation of isolated intact parenchymal cells from rat liver. *J Cell Biol 35:675-684, 1967*

Kinosita R: Reseraches on the carcinogenesis of the various chemical substances. *Gann 30:423-426, 1936*

Kotin P: Dose-response relationship and threshold concepts. *Ann N V Acad Sci 271:22-38, 1976*

Kurth R, Fenyo EM, Klein E, Essex M: Cell surface antigens induced by RNA tumor viruses. *Nature 279:197-201, 1979*

Lijinsky W, Taylor HW: Relative carcinogenic effectiveness of derivatives of nitrosodiethylamine in rats. *Cancer Res 38:2391-2394, 1978*

Lim I-K, Hwang DS, Kim YS: A study of plasma membrane surface proteins of transformed rat liver cells induced by 3'-methyl-4-dimethylaminoazobenzene. *Yonsei J Med Sci 15:308-323, 1982*

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem 193:265-275, 1951*

Mainigi KD, Sorof S: Evidence for a receptor protein of activated carcinogen. *Proc Natl Acad Sci 74:2293-2296, 1977*

Meltzer MS, Leonard EJ, Rapp HJ, Borsos T: Tumor-specific antigen solubilized by hypertonic potassiium chloride. *J Natl Cancer Inst 47:703-709, 1971*

Miller EC, Miller JA: The presence and significance of bound aminoazo dyes in the livers of rats fed p-dimethylaminoazobenene. *Cancer Res 7:468-480, 1947*

Miller JA: Carcinogenesis by chemicals: An overview-G.H.A. Colwes Memorial Lecture. *Cancer Res 30:559-576, 1970*

Miller JA, Miller EC: The carcinogenicity of certain derivatives of p-dimethylaminoazobenzene in the rat. *J Exp Med 87:139-156, 1948*

Natori T, Law LW, Appella E: Biological and biochemical properties of Nonidet p40-solubilized and partially purified tumor-specific antigens of the transplantation type from plasma membranes of a methylcholanthrene-induced sarcoma. *Cancer Res 37:3406-3413, 1977*

O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. *J Biol Chem 250:4007-4021, 1975*

Opie EL: The pathogenesis of tumors of the liver produced by Butter Yellow. *J Exp Med 80:231-246, 1944*

Orr JW: The histology of the rat's liver during the course of carcinogenesis by Buffer Yellow (p-dimethlaminoazobenzene). *J Path Bact 50:393-408 1940.*

Ouchterlony O: Antigen-antibody reaction in gels. *Acta Pathol Microbiol Scand 26:507, 1949*

Patel NT, Folse DS, Holoubek V: Release of repetitive nuclear RNA into the cytoplasm in liver of rats fed 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res 39:4460-4465, 1979*

Patel NT, Yoshida M, Holoubek V: *In vitro* incoporation of 3'-methyl-4-dimethylaminoazobenzene into liver nuclei and release of RNA from the nuclei. *Cancer Res 41:743-751, 1981*

Pellis NR, Shulan DJ, Kahan BD: Specific tumor immunity induced with soluble materials: Purification of antigens inducing tumor resistance. *Biochem Biophys Res Commun 71:1251-1258, 1976*

Peraino C, Fry RJM, Staffeldt E: Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. *Cancer Res 31:1506, 1971*

Richardson HL, Nachtnebel EB; Study of liver tumor development and histologic changes in other organs in rats fed azo dye. *Cancer Res 11. 398-403, 1951*

Sorof S, Young EM: The principal liver carcinogen protein conjugate after a single dose of hepatic azocarcinogen. *Cancer Res 33:2010-2013, 1973*

Sorof S, Young EM, McBride RA, Coffey CB: On protein targets of chemical carcinogens: Dissimilar molecular sizes of the principal pretein conjugates *Cancer Res. 30:2029-2034, 1970*

Stevens RH, Cole DA, Cheung HF: Identification of a common oncofetal protein in X-ray and chemically induced rat gastrointestinal tumors. *Br J Cancer 43:817-825, 1981*